Effects of trace metals on cellular immune responses, tissue injury and gene expression in the mussel, *Mytilus edulis*: implications for biological monitoring of marine pollution.

by

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Abstract

The immunotoxic effects of trace metal exposure in bivalves are poorly understood and whether or not stimulation of the immune system exacerbates metal toxicity is still unclear. The mussel, *Mytilus edulis*, was exposed to 50 or 500 $\mu g\ l^{-1}$ total Hg or 20 or 50 $\mu g\ l^{-1}$ total Cd for up to 11 days compared to no added Hg or Cd controls. Selected experiments were then repeated in mussels injected with lipopolysaccharide (LPS) to challenge the immune system with or without exposure to 50 $\mu g\ l^{-1}$ Hg or 20 $\mu g\ l^{-1}$ Cd for 8 days. Immune functions of haemocytes, haematology, haemolymph glucose, electrolytes, superoxide dismutase (SOD) activity and organ pathology were measured. Hg accumulated mainly in gills and Cd in digestive gland. Hg or Cd exposure alone caused transient modulation of haemocyte immune functions, and haemolymph Na, K and glucose levels were negligible. LPS injection had a significant effect on haemocyte numbers and variable response patterns in phagocytosis and neutral red uptake (NRU) compared to other mussel groups. Tissue pathology was shown in Hg and Cd exposures and greatly increased by the effect of LPS injection. In conclusion immunostimulation with LPS can increase Hg or Cd-related organ pathologies, but not necessarily alter the responses of haemocytes. Mussels collected from field are widley used to assess the effects of pollution on the aquatic environment, and the effect of pre-exposure history on the immunological response to subsequent metal exposure is poorly understood. The current work aimed to compare the health status in animals from a clean to a contaminated site in South West England to determine whether these end points could be useful in biomonitoring. *Mytilus edulis* were collected from both sites and analysed for trace metals, cellular immune responses, biochemistry, and condition index (CI) and

histopathology. Groups of mussels from each site were exposed to 20 $\mu g\ l^{-1}$ $CdCl_2$ for up to 8 days compared to unexposed controls. End points were tissue and hamolymph metals/electrolytes, cellular immune functions and histology. For field work, concentrations of Cd, Cu, Fe, Zn and Pb were significantly higher in mussels from the polluted site. A strong correlation between shell length and metal concentration was observed. Pollution had an effect on NRR, (CI), and tissue pathology of polluted site mussels. For the laboratory study, immune functions, CI and Na or K levels did not follow clear trends in site or Cd exposure effects. Tissue necrosis and inflammation in animals from the polluted sites were shown, but Cd exposure improved some of these pathologies. I conclude that metal pollution modulates biological functions in *M. edulis*, but pre-exposure history has limited effects on these responses. Tissue histology is a sensitive tool to distinguish clean from polluted sites.

Copyright statement

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Chapter 1

Introduction and literature review

The effects of pollutants on the aquatic invertebrates

Pollution is the direct and indirect introduction of substances, or energy (e.g. thermal pollution), to the aquatic environment. Pollution may result in deleterious effects on organisms and ecosystems, hazards to human health, and adverse effects on human use of water resources including fishing, water sports, as well as drinking water abstraction (Maduka, 2006). Pollutants/chemicals rarely occur in isolation, but are in mixtures of several substances in the environment and this may result in interactions where the resulting toxicity may be greater than the sum of the parts, or the biological effects may be complex (El-Masri et al., 1995; Malmqvist and Rundle, 2002; Duchemin et al., 2008). For metals, perhaps the most well known effects are on salt and water balance in aquatic organisms (review, Reader et al., 1989; Wood, 1992; Rainbow and Black, 2005). However, environmental pollutants such as trace metals exert a range of biological effects including effects on immunity (Sengupta and Bishayi, 2002; Fisher, 2004), respiration and metabolism (Brown and Newell, 1972; Engel and Fowler, 1979).

Traditionally, metal toxicology has considered whether or not the metal of interest is a biologically essential metal like Cu, Fe, Mn and Zn; or non-essential such as Cd or Hg (Jensen and Bro-Rasmussen 1992; Wang et al., 2004). It is well known that trace metal exposure is a function of free metal ion chemistry in the water and subsequent bioavailability (Rainbow and Black, 2002), with uptake via the food (Handy et al., 2005) or water (Łobiński and Szpunar, 1999; Boening, 2000) causing metal accumulation in organisms (Rainbow and Black, 2002). Sub-lethal metal toxicity can cause a reduction in ecological competitiveness of the affected species, and this is not necessarily a direct effect of metal toxicity *per se* (by itself), but indirect effects including disease, parasitism and increased risk of predation (Fournier et al., 2000). The end result in ecological terms may be a progressive change in the population size of the

affected species. Some reports have considered the importance of immunotoxicity in this process, and it has been suggested that changes in immunity caused by metal exposure may be a key factor in the chronic effects of metal pollution (Galloway and Depledge, 2001; Morley et al., 2003).

1. The importance of mussels

Mussels are filter feeders and can filter up to 96 litres of water a day (Grant et al., 2005). Thus, they can accumulate relatively large concentrations of contaminants as water passes through their gills, and this can include particulate forms as well as dissolved metals. Therefore, they are often used as sensitive organisms to evaluate pollution in aquatic environments (Goldberg, 1989; Livingstone et al., 2000; Rickwood and Galloway, 2004; Romeo et al., 2005; Vidal-Liñán et al., 2010).

Bivalve molluscs belonging to genus *Mytilus* are distributed throughout the world and are widely used as biomonitoring organism. The health condition of marine mussels is used as a bioindicator of water quality in coastal areas (e.g., Svärdh and Johannesson, 2002; Wootton et al., 2003). The marine mussels *Mytilus edulis* and *Mytilus galloprovincialis* are also a commercially valuable species.

1.1 Routes of exposure and target organs for metals in bivalves

Metals are probably absorbed from the environment in several ways. The contamination may be direct by water or indirect by food. Mussels are filter feeders and ingestion of phytoplankton or other suspended particles in *Mytilus* spp. is well documented (George, 1980). Clearly, food route exposure is a concern. High concentrations of metals can also be found in aqueous solution and sediments (Temara et al., 1997; Sauvé et al., 2002) and so aqueous exposure via the gills is also possible. Some tissues, like the gills, are involved in the uptake mechanisms, which may involve

metals being bound to particulate materials or in a soluble state (Irato et al., 2003). The target organs for metals, therefore, can include the mantle, gills, digestive gland, reproductive tissue, and sometimes the adductor muscle (Soto et al., 1997; Bolognesi et al., 1999; Romeo et al., 2005; Kljaković-Gaspić et al., 2006).

1.2 Immune system of invertebrate organisms

The major function of the immune system in all organisms is to supply protection against infectious agents (bacteria, viruses, fungi, yeast and parasites), to reject non-self components, and to be involved in the inflammation process that initiate tissue repair. This is achieved by a system of recognition, to distinguish self from non-self, and response to eliminate invading organisms or foreign particles (Gliński and Jarosz, 2007).

Molluscs possess an open circulatory system, which is continually exposed to fluctuations in environmental factors including contaminants (Cheng, 1981). The immune system of bivalve molluscs relies on circulating haemocytes present in the blood, which are able to travel throughout the tissues in response to non-self materials, including latent pathogens. The mussels' immune defence is comprised of innate and humoral mechanisms, in which haemocytes play a key role (Canesi et al., 2002). A natural immune system has been discovered in bivalves, formed by outer and internal protective defences. The internal defence mechanisms include cellular reactions such as: phagocytosis, encapsulation, and melanisation. Humoral defences are formed of lysozyme activity, lectins, antimicrobial agents and the reactive oxygen metabolites Figure (1.1).

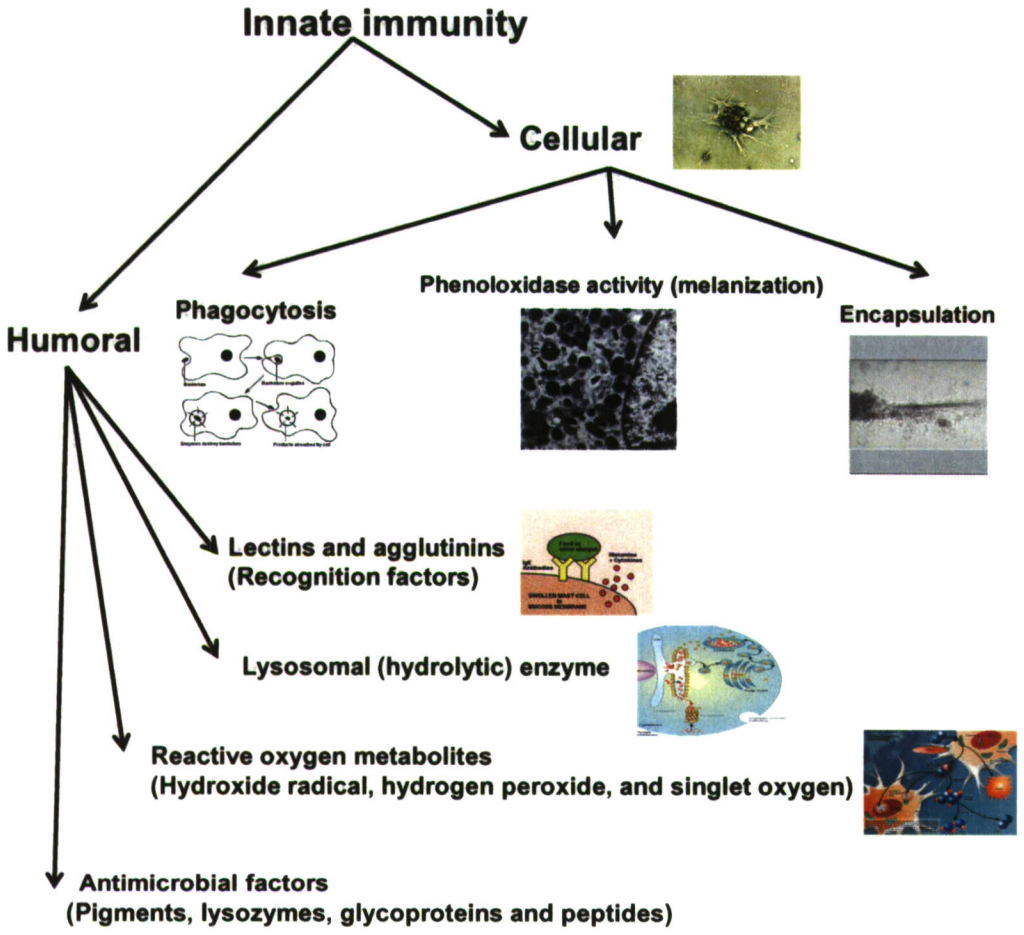

Fig. 1.1 Schematic diagram describes the cellular and humoral components of the innate immune system in bivalve molluscs

1.2.1 Defensive barriers

As the first line of defence, anatomical and physio-chemical barriers to potential pathogens and parasites include the epidermis and perhaps the cuticle (exoskeleton), shell and/or mucous layer found in different invertebrates. They prevent damage of the underlying tissues and the loss of body fluids. The integrity of body coverings is supported by blood clotting and wound healing processes (Glinski and Jarosz, 1997). Some studies have been done on the effect of pollutants on the defensive barriers of molluscs. Mussels can avoid contaminant exposure by shell clamping, a protective mechanism that has been identified in *M. edulis* when subjected to organophosphate (OP) pesticide contamination (Mohan et al., 1987). Aldridge et al. (2006) demonstrated that zebra mussels are able to sense chlorine and other toxins in their surrounding environment and respond by closing their valves, thus enabling them to avoid toxic effects for up to 3 weeks.

Sindermann (1993) reported that, bivalve molluscs associated with contaminant exposure (organotin compound such as antifouling paint used on boats) for 5 years showed shell atrophy in the British east coast. Mucus secretions are also able to chelate trace metals and reduce exposure (see Handy and Maunder, 2009 for a review of mucus), and are an important barrier in molluscs (Iguchi et al., 1982; Simpkiss, 1990)

1.2.2 Nacrezation

Nacrezation is the deposition of nacre around invading parasites, and it is deposited in response to irritation or infection of the mantle region (Cheng, 1967; Laruelle et al., 2002). Nacre is made of organic-inorganic mineral deposits, and its normal biological function is the creation of pearls, which indicates that the mantle is the organ responsible for pearl formation, and Cremonte (2004) found that the mantle secretes

nacre deposits around the trematode parasite (*metacercariae*) as part of a defence mechanism.

1.2.3 Types of haemocytes in bivalves

Cuénot (1891) suggested that, the origin of haemocytes might be in special "gland lymphatiques" at the base of the gills. Morton (1969) documented that some haemocyte formation in *Dreissena polymorpha* was related to the circadian rhythm of the adductor muscle. Cheng (1996) suggested that haemocytes of the eastern oyster *Crassostra virginica* arise from differentiation of connective tissue cells.

In an earlier research by Moore and Lowe (1977), classification of *M. edulis* haemocytes relying on structure, function and staining properties was done. They divided the haemocytes into two main categories: basophilic and eosinophilic haemocytes (granulocytes). The basophilic cells were divided again into small lymphocytes and larger phagocytic macrophages with lysosomal enzymes reactivity, where the latter were the main cell in particles uptake. However, Cajaraville et al. (1995) classified haemocyte in bivalves into two main groups: granular and agranular, and both are able to take up particles. The haemocytes of *Mytilus californianus* were classified into three types: small and large basophils and large granular acidophils (Bayne et al., 1979). They found that both acidophilic and basophilic haemocytes have the ability to phagocytose particles (Agglutinins and human A Rh +ve erythrocytes) *in vitro*. The most widely accepted classification scheme for bivalves based on morphological differences is that presented by Cheng (1981). The classification included three cell types: 1) hyalinocytes, which are cells with nearly clear cytoplasm containing few or no granules; 2) granulocytes (granular haemocytes), which are large actively phagocytic cells containing numerous acidophilic cytoplasmic granules, and 3) serum cells, which are tightly packed with many pigment globules.

Differential staining methods are generally used in haemocyte characterization. According to the staining there are two categories of haemocytes; acidophilic (eosinophilic) which appear pink, and basophilic populations which appear blue following different staining techniques (Zhang and Wang, 2006) According to Pipe et al. (1997); and Wootton et al. (2003), basophilic haemocytes of *M. edulis* consisted of both agranular and granular cells which are eosinophilic cells. Electron microscope studies confirm that cells containing large-sized granules (ca. 10-12 and 7-8 μm diameter) are eosinophilic and small-sized agranular cells are basophilic (ca. 2-4 μm diameter). Glinski and Jarosz (1997) reported that the granular haemocytes are the most numerous cell type of molluscan blood that is active in cellular defence. Wootton et al. (2003) stated that granular eosinophilic haemocytes have been shown to be the most active in terms of phagocytosis release of reactive oxygen metabolites and lysosomal enzymes, and for uptake of neutral red. They also demonstrated high levels of activity for phenol oxidase and peroxidase. Table 1.1 summarises the different types of cells responsible for the immune functions in some aquatic invertebrates.

Table 1.1 Types and functions of some immune cells from aquatic invertebrates

Class/Phylum	Cell type	Function	References
Polychaeta	Amoebocytes and coelomocytes	Phagocytosis and encapsulation	Smith (1950)
Gastropoda	Amoebocytes	Phagocytosis	Anderson (1977)
Bivalvia	Granulocytes (eosinophils)	Phagocytosis, lysosomal enzymes and production of ROS	Carballal et al. (1997); Wootton et al. (2003)
Echinodermata	Amoebocytes and spherule cells	Encapsulation	Johnson (1969)

1.3 Cellular responses to immune challenge

1.3.1 Phagocytosis, encapsulation and melanisation

The initial response of molluscs to wounding or infection involves the infiltration and accumulation of phagocytic haemocytes at the site of injury (Baker, 1976). The evaluation of the phagocytic capacity of bivalve haemocytes is particularly important in the estimation of defence capabilities because most of the clearance and intracellular destruction of microoganisms is carried out by these cells (Rodrick and Ulrich, 1984). Torreilles et al. (1996) and Lopez et al. (1997) suggested that the process of phagocytosis may be characterised by the following steps: (1) Chemotactical activation of haemocytes; (2) attachment of particles to the surface of the phagocytes; (3) incorporation of the particles and (4) their intracellular digestion. Moreover, phagocytosis is accompanied by the release of a variety of cytotoxic agents (reactive oxygen species, ROS and peroxidase which are involved in the oxidative burst) and lysosomal enzymes including esterase (Pipe, 1990). This process involves the migration of haemocytes toward substances released by damaged tissues and/or infecting organisms.

Several authors have demonstrated that the granulocytes in bivalves were the most abundant and actively phagocytic cells which constituted between 70 - 93 % of the total haemocyte count (Lopez et al., 1997; Pipe et al., 1997; Wootton et al., 2003). Phagocytosis is an energy dependent process and is sensitive to intracellular energy levels (Sokolova et al., 2004). Phagocytosis requires motility of the cell membrane and this process uses significant amounts of ATP for cytoskeletal rearrangement through the actomyosin system (Wells et al., 1999). The synthesis of intracellular protein involved in this process, their subsequent transport and the insertion of membrane receptors are compromised by reduced intracellular energy levels (Karlsson and Nassberger, 1992). Phagocytic haemocytes of invertebrates produce interleukin-1 (IL-1). IL-1 is also the

prototypic "alarm" cytokine in that it brings about increases in a variety of defence mechanisms, particularly immunologic and hematologic responses (Hughes et al., 1991). IL-6 and tumour necrosis factor (TNF)-like molecules as well as other regulatory cytokin-like products (e.g. DNA-binding protein responsible for IL-1-stimulated IL-6 induction and multifunctional cytokines involved in the regulation of the immune response, haematopoiesis, and inflammation (Bloc et al., 2002).

Phagocytosis of larger particles or large numbers of pathogens that cannot be ingested by single phagocyte is achieved by encapsulation or cell clumping/nodule formation (absorbing large molecules through their outer cell membrane) in bivalves (Cheng, 1984; Tripp, 1992) and in other molluscs and other phyla (Glinski and Jarosz, 1997; Galloway and Depledge, 2001). Wootton et al. (2006) reported that encapsulation is the common immune defence reaction towards foreign bodies in invertebrates, including multicellular parasites, which enter the heamocoel and are too large to be phagocytosed. Suzuki and Mori (1990) discussed the recognition process is generally mediated by specific or non-specific substances such as lectins or opsonins (Yang and Yoshino, 1990).

Jiravanichpaisal et al. (2006) discussed the functions of haemocytes in innate immune responses and their roles in melanisation in arthropods. The darkening of blood on exposure to air is due to the oxidation of haemolymph diphenols by the action of phenol oxidase in the haemocytes, this process is called melanisation (Ratcliffe and Rowley, 1981). Melanin is involved in wound healing and melanisation of pathogens (infestation) and physical damage in tissues and immune responses. Phenol oxidase is a key enzyme responsible for the catalysis of the melanization reaction in invertebrates (Cerenius and Soderhall, 2004; Procházková et al., 2006). The pro-phenol oxidase activation system controls the deposition of melanin around damaged tissue or intruding objects, mainly in insects and crustaceans (Cerenius and Soderhall, 2004). The

prophenol oxidase system is an enzyme cascade activated by contact with bacterial components analogous to the vertebrate complement system and involved in non-self recognition (Soderhall et al., 1997). Phenoloxidase activity was observed in haemocytes and haemolymph of the marine bivalve *Perna viridis*, playing an important role in non-self recognition and host immune responses (Asokan et al., 1997). Serine proteases play an important role in the activation of prophenoloxidase (proPO), a critical enzyme in the production of melanin in insects (Paskewitz et al., 2006).

1.4 Humoral responses to immune challenge

Cell killing has been demonstrated in haemocytes of bivalves during the phagocytic process: (i) lysosomal enzymes and other lysing agents release from haemocytes to haemolymph (Cheng, 1981; Mohandas et al., 1985), and (ii) the release of reactive oxygen metabolites from the haemocytes as a result of a respiratory burst into serum (Pipe, 1992; Arumugam et al., 2000).

1.4.1 Lysosomal enzymes

Lysosomes are sub-cellular organelles bounded by a semi-permeable lipoprotein membrane containing a battery of 60 hydrolytic enzymes. Their optimal pH is acidic, around pH 4-5 (Lowe and Fossato, 2000). Bivalve lysosomes are sites of intense intracellular digestion. Cheng (1981) documented that lysosomal enzymes within the phagocytes degrade non-self particles (which have been phagocytosed by the haemocyte). In some cases lysosomal enzymes have been released from haemocytes into the plasma or extracellular compartments of other tissues to degrade foreign material. The release of lysosomal enzymes by granular haemocytes is visually observed as degranulation of the cell (Mohandas et al., 1985). The lysosomal enzymes

released can include: acid phosphatase; β-glucuronidase, non-specific esterases, arylsuphatase and N-acetyl-β-hexosaminidase.

Lysozyme is a lysosomal enzyme with an important defence role, since it can hydrolyze components of bacterial cell walls. It also participates in digestion, because bacteria are a part of the diet of many marine bivalve molluscs (Cheng, 1983). Cheng (1986) reported that elevated levels of lysosomal enzymes in serum also contributed to the inflammatory response. Many studies using enzyme cytochemistry have demonstrated certain lysosomal enzymes associated with the granules of the haemocytes (Cheng, 1981; Cheng, 1984; Pipe, 1990; Lopez et al., 1997), and this led to the conclusion that the haemocyte granules are a form of lysosome.

1.4.2 Reactive oxygen species (ROS)

Mitochondria have been documented as an important site for ROS production (Boveris, 1984). ROS are products of the respiratory burst in haemocytes, and can act as mediators of both oxidative damage and immune-related intracellular signalling pathways. Increased rates of oxygen uptake are a result of phagocytic cell membrane stimulation. This respiratory burst is associated with production of superoxide anions ($O^{\cdot}_2$) via membrane-bound NADPH-oxidase (Chanock et al., 1994). Subsequent reactions (Haber-Weiss, Fenton reactions) result in additional ROS such as: hydrogen peroxide (H_2O_2), hydroxide radical (OH), and singlet oxygen ($^1O_2$) (Halliwell and Gutteridge, 1990). Bivalve haemocytes are capable of an 'oxidative or respiratory burst' releasing ROS (Goodal et al., 2004). Cajaraville (2003) also argues for ROS causing DNA damage in haemocytes.

Reactive nitrogen intermediates including nitric oxide (NO) have been identified in relation to phagocytosis in molluscan haemocytes (Torreilles and GuÉRin, 1999). Nitric oxide is a well-known gas and it is important as a signalling molecule which is

synthesized from L-arginine by the enzyme nitric oxide synthase. It has been identified in various vertebrate tissues. In mammals, the effect of nitric oxide is to control the reduction state of the electron transport by lowering cytochrome oxidase O_2 affinity (Abele et al., 2007). Production of nitric oxide in invertebrates (cultured bivalves and crustaceans) hemocytes has been demonstrated by Roch (1999). The most common sites for the production of nitric oxide are reported in the central and peripheral nervous systems. The functional roles of nitric oxide in invertebrates are strongly connected to the physiological activities described in vertebrates, defence, neurotransmission, salt, water balance and immunity (Martinez, 1995; Coleman, 2001).

1.4.3 Antibacterial activity

A variety of antimicrobial factors, like pigments (Sanduja et al., 1985), lysozymes (Nilsen et al., 1999), glycoproteins (Yamazaki, 1993) and peptides (Iijima et al., 2003) have been isolated from molluscs. Two modes of action have been detected by these materials: (a) disruption of the bacterium negatively charged cytoplasmic membrane or, (b) killing bacteria without membrane lysis, involving the binding of antimicrobial peptides to a specific protein following translocation into the bacterium (Brogden, 2005).

Antimicrobial (antibiotic) peptides are small molecules and essential elements of the innate immune defence against fungal and bacterial infections. According to their primary structure, more than 1000 antimicrobial peptides have been identified (Bulet et al., 2004) and these are characterized by a predominance of cationic and hydrophobic amino acids. Brogden et al. (1996) have also identified peptides with anionic properties. This amphipathic (anionic and cationic properties) structure allows them to interact with the bacterial membrane and the host cell. In vertebrates, they have strategic locations in phagocytes, in body fluids, and on epithelia, enabling these antimicrobial defences to

interface with the external environment (Tasiemski et al., 2004). However in invertebrates, these peptides have been detected in the haemolymph of bivalves including oysters (Anderson and Beaven, 2001) and in haemocytes of the mussels (Mitta et al., 1999). Recently, Gueguen et al. (2006) isolated antimicrobial peptides from the mantle of the oyster *C. gigas*.

Antimicrobial peptides (proteins and glycoproteins) are pivotal elements of the innate immune defence against bacterial and fungal infections and have been detected in the digestive tissues of various molluscs (Iguchi et al., 1982; Pakrashi, 2001). Haug et al. (2004) found antibacterial activity in extracts from several tissues, including plasma haemocytes, labial palps, byssus, mantle, and gills in the horse mussel, *Modiolus modiolus* from Norway. They also found lysozyme like activity and toxic activity against *Artemia salina* in fractions from the gills, mantle, muscles and haemocytes and argued that the antibacterial factors might therefore have an important function as a first line of defence against pathogenic microorganisms. Antimicrobial peptides have been isolated and characterised from the haemocytes of *M. edulis*, two isoforms of a novel 34-residue, cysteine-rich peptides with potent bactericidal activity. They were named mytilins and appeared more active against Gram-positive bacteria than Gram-negative bacteria (Charlet et al., 1996; Mitta et al., 2000a).

1.5 Immunotoxicity

Assays to determine the immune function may provide a sensitive measure of the health status of an individual or population under pollutant stress. As the haemocytes in mussels take part in the elimination of foreign particles (through phagocytosis), these cells are considered to be particularly well suited as indicators of health (Weeks et al., 1992). Lysosomes accumulate many pollutants to high concentrations resulting in membrane destabilisation. Consequently, the clarification of lysosomal membrane

integrity has been used as a good effect in pollution monitoring (Nicholson, 2001). Concern is increasing regarding the possible adverse effects of chemicals on the immune system of humans and wildlife. Many classes of environmental pollutants have been implicated in the modulation of immune function, including trace metals (Fairbrother, 1994; Pipe and Coles, 1995; Galloway and Depledge, 2001). There are growing observations of metal-induced immunotoxicity and disease conditions in marine organisms. Bengsch (1972) documented that the greater the pollution load the greater the concentration of potential infection organisms in the bivalve molluscs. Pipe and Coles (1995) presented data showing the effects on immune parameters of the mussels exposed to *Vibrio tubiashi* following pre-exposure to copper or cadmium, are examples.

Environmental pollutants such as trace metals exert a range of immunotoxicological effects on aquatic organisms (Table 1.2). Significant destabilisation of the haemocyte lysosomal membrane and impaired phagocytosis has been approved as an effect of metal pollution such as As, Cd, Cu, Hg, Pb, and Zn in mussels (Brousseau et al., 1999; Olabarrieta et al., 2001; Galloway et al., 2002; Gomez-Mendikute and Cajaraville, 2003; Nicholson, 2003; Marchi et al., 2004). A wide range of metal effects has been documented to cause haemocyte mortality, modulate haemocyte numbers and their degenerative enzymes in bivalves (Coles et al., 1995; Parry and Pipe, 2004; Gagnaire et al., 2004).

Table 1.2 The immunotoxic effects of some toxic metals on bivalve molluscs

Toxic metals	Conc. and exposure time	Organisms	Immunotoxic effect	References
As, Cd, Cr, Cu, Hg, Ni, Pb, Se and Zn	Field survey	Ribbed mussel *Geukensia dermmissa*	Decrease of lysosomal stability, cell viability and phagocytosis ability of haemocytes.	Galloway et al. (2002)
$CdCl_2$ and $CuCl_2.2H_2O$	Up to 1120 Cd and up to 12.72 Cu ($x10^5$) $\mu g\ ml^{-1}$, for 24 h.	The common mussel *M. galloprovincialis*	2.25 fold decrease in neutral red uptake and disrupted actin cytoskeleton in mussels` haemocytes.	Gomez-Mendikute and Cajaraville (2003)
CuSO4	Up to 200 $\mu g\ l^{-1}$, for up to 72 h.	The green mussel *Perna viridis*	Destabilisation of haemocyte lysosomal membrane and impaired phagocytosis.	Nicholson (2003)
$CdCl_2$	40 and 400 $\mu g\ l^{-1}$, for 7 days.	The blue mussel *Mytilus edulis*	Decreased haemocytes numbers to 5.7 x 10^6 cell ml^{-1}, increased uptake of neutral red to 3.75 flod and 1.7 fold for degradative enzymes.	Coles et al. (1995)
$CdCl_2$, $HgCl_2$ and CH_3Hg	3 pM - 0.3 mM $CdCl_2$, 0.2 μM - 0.2 mM $HgCl_2$ and 2 pM - 2 μM CH_3Hg, for 4/24 h.	Pacific oyster *C. gigas*	Increase of haemocytes mortality to 80%, esterase, peroxidase, aminopeptidase and phagocytosis.	Gagnaire et al. (2004)
$CdCl_2$	0.01 - 2 mM, for 24 h	The common mussel *M. galloprovincialis*	Stimulate phagocytic and lysosomal activities in haemocytes for up to 2 folds.	Olabarrieta et al. (2001)
$ZnCl_2$, $CdCl_2$, AgNO3, $HgCl_2$, CH_3HgCl	10 pM - 10 mM , for 18 h	*Mya arenaria*	Suppression of phagocytosis related to a decreased cell viability.	Brousseau et al. (1999)
CuSO4	0.02 and 0.05 $\mu g\ l^{-1}$ for 7 days.	The blue mussel *Mytilus edulis*	Inhibition effect on total haemocyte counts to 2.68 x10^6 cell ml^{-1}. Affected production of intracellular superoxide dismutase and phagocytosis.	Parry and Pipe (2004)
Hg^{2+}	50 μM for 60 min.	The common mussel *M. galloprovincialis*	Reduced NR retention by 30 %.	Marchi et al. (2004)

1.6 Histopathological effects of pollution on bivalves

Biomarkers are defined as a biological response to chemical(s) that give a measure of exposure or effect (Peakall, 1994). However, Hinton et al. (1992) defined biomarkers in general as any contaminant induced physiological/chemical changes in organisms that leading to the development of cell/tissue/organ lesions. They defined histopathological biomarkers as the lesions (eg., necrosis) cellular toxicity and also reductions in host defence resulting from effects of exposure to one or a mixture of toxicant(s). Histopathology is considered the most rapid method of detecting adverse acute or chronic exposure effects in many tissue organs of fish and bivalve molluscs (Hinton et al., 1992). Tissue histopathology now is widely used as a biomarker to asses chronically exposed environments (David et al., 2008 b). Handy et al. (2003) documented histological biomarkers as an approach which meets the criteria of chronic biomarkers of effect and exposure. Handy et al. (2003) described histological biomarkers as a successful chronic biomarker because of its attributes which included detecting long-term effects ranging from days to years, involving different body cell types and with have biochemical memory, and can able to show negligible effects of temporal variability, and remain effective regardless of changes in body burden distribution. The biomarker response should be inducible for a long time and should tolerate short-term variability in exposure.

Some contaminants were been found to be tissue specific in their effect. Some pollutants concentrate in gonadal tissue (McCormick et al., 1989) and non-gonadal tissue (Cunningham and Tripp, 1975). A correlation between tissue lesions and body burdens in the Asian clam, *Potamocorbula amurensis*, from San Francisco Bay was demonstrated in a study by Clark et al. (2000). The Mussel Watch Program relies mainly on histological examination to determine a wide range of parasites, pathology and diseases. Histological grading has been suggested as a measure of overall health in

the wild and transplanted mussel *Dreissena polymorpha* (Bowmer et al., 1991). Morphological characters (eg., organelle, cell number, volume and organ weight) can be used as integrators of physiological and biochemical responses. In addition, histological sections have the ability to spot alterations in specific cell types with specific functions, and which cell undergo necrosis before the death of the animal whether than using the whole organ homogenates for enzyme markers (Hinton et al., 1992).

The immune organs of invertebrates are diffuse and in many tissues, and the toxic effects on organs may reveal concerns about the integrity of the immune system in general. Histopathological scouring was applied as a measurement of health in tissue (Weibel et al., 1966) including mussel tissue exposed to contaminants and this information can be used successfully in the comparison of field samples (Auffret, 1988; Bowmer et al., 1991). Hinton et al. (1992) argued that histology has the greater utility as a biomarker that can be applied in the field of chronic monitoring, because of its ability to detect parasite prelevance (Auffret, 1988), nutritional status (Abi-Ayad and Kestemont, 1994) seasonal changes (Leatherland, 1970), and sex differentiation (Cooke and Hinton, 1999).

Histopathological studies show a variety of organ damage in bivalve molluscs exposed to metals, including tissue inflammation. Rasmussen et al. (1983) examined acute (up to 0.8 mg for 15 days) and chronic (0.2 mg for 8 weeks) effects of N-nitroso compounds on tissues of the blue mussel, *M. edulis*. They found tissue alterations characterised by extensive inflammatory reactions in the Leydig (interstitial) cell tissue, necrosis in the epithelial lining of the digestive tubules, collagenous scar tissue formation in between the tubules of the digestive gland, encapsulation or replacement of damaged digestive tubules by collagenous scar tissue, necrosis in the germinal epithelium lining the genital ducts and the presence of numerous granulocytoma. Teh et al. (1999) studied histopathologic alterations of the digestive gland, gill, gonad, and

kidney in the Asian clams (*Potamocorbula amurensis*) from each of four United States Geological Survey (USGS) stations in the San Francisco Estuary based on body burdens of metallic contaminants in clams observed over 7 years. They found mild digestive gland atrophy, moderate kidney tubular atrophy, and moderate gill inflammation in the collected clams that were correlated with tissue metal levels.

The gills and digestive gland of the common mussel *M. galloprovincialis*, showed histological changes after exposure to heavy metals (0.1 mg l^{-1} of Hg, Ag, Pb, Cu for 98 days). The exposure resulted in fusion of gill filaments and presence of residual bodies in the digestive cells, fragmentation or vacuolization of the rough endoplasmic reticulum, and increase in the number of granules in the basophilic cells (Domouhtsidou and Dimitriadis, 2000). Usheva et al. (2006) studied the histomorphological effects of the polluted waters of the Tumannaya River Russia on the mussels *Crenomytilus grayanus*. Granulocytoma were a feature of connective tissue with necrosis and lysis, the tubule` epithelium was characterised with erosion and vacuolization of digestive cells and swollen nervous fibres of the gland have been observed.

Data on histopathology related immunotoxic effects of trace metals in invertebrates and mammals are rare. Most of the work has been done on fishes and effects of metals on the immunity and related target organs. Aims of the field or experimental work in invertebrates always concentrate on the specialized area of immunity biomarkers or histology techniques separately, but not combined or integrated biomarkers of immunity and histology. Table 1.3 explores some of the papers on fish and the only work has been done on immunity in relation to tissue pathology under the effect of trace metals in bivalves.

Table 1.3 Histological alterations related to immunotoxic effects of some trace metals in aquatic organisms

Trace metals	Conc. and exposure time	Immunotoxic/histopathologic effect	References
$HgCl_2$	50 $\mu g\ l^{-1}$ for 11 days	Hg modulated immunity which caused tissue pathologies of *M. edulis*	Sheir et al. (2010)
$CdCl_2$	Up to 50 $\mu g\ l^{-1}$ for 11 days	Immunostimulation greatly increase Cd-related organ pathologies of *M. edulis*	Sheir and Handy (2010)
CuSO4	Up to 175 $\mu g\ l^{-1}$ for 96 h	Correlation between the degree of gill hyperplasia and the macrophage chemiluminescence response in goldfish	Muhvich et al. (1995)
$HgCl_2$ and CH_3HgCl	Up to 100 mg Hg kg^{-1} DW, for 4 months	Lipid peroxidase increase and brain lesions were accompanied in Atlantic salmon	Berntssen et al. (2003)
Combined (Cd + Pb + Cr + Ni)	5 mg l^{-1} for 32 days	Macrophage reactions approved in the affected kidney tissue of the common carp	Vinodhini and Narayanan (2009)

1.7 Hypothesis

The main hypothesis is that Hg and Cd interfere with immunity in bivalves. Hg and Cd accumulate mainly in the gills and digestive gland, respectively. They disturb electrolyte exchange through affecting cell membrane permeability. So, the latter event will interfere with the cell signalling cascade of the cell. Gene expression will be modulated and react by increasing metallothionein induction. Toxicity of metals could be a result of the spillover of free metal ions in the cell more than metals bound to metallothionein like-proteins, or binding to active sites of proteins and enzymes by inhibiting them. Neurotxicity of metals can be generated by respiratory burst (ROS) production. Consequently, immune functions will be inhibited and tissue pathology will appear as a result of ROS production if not enough antioxidants are produced (Fig. 1.2).

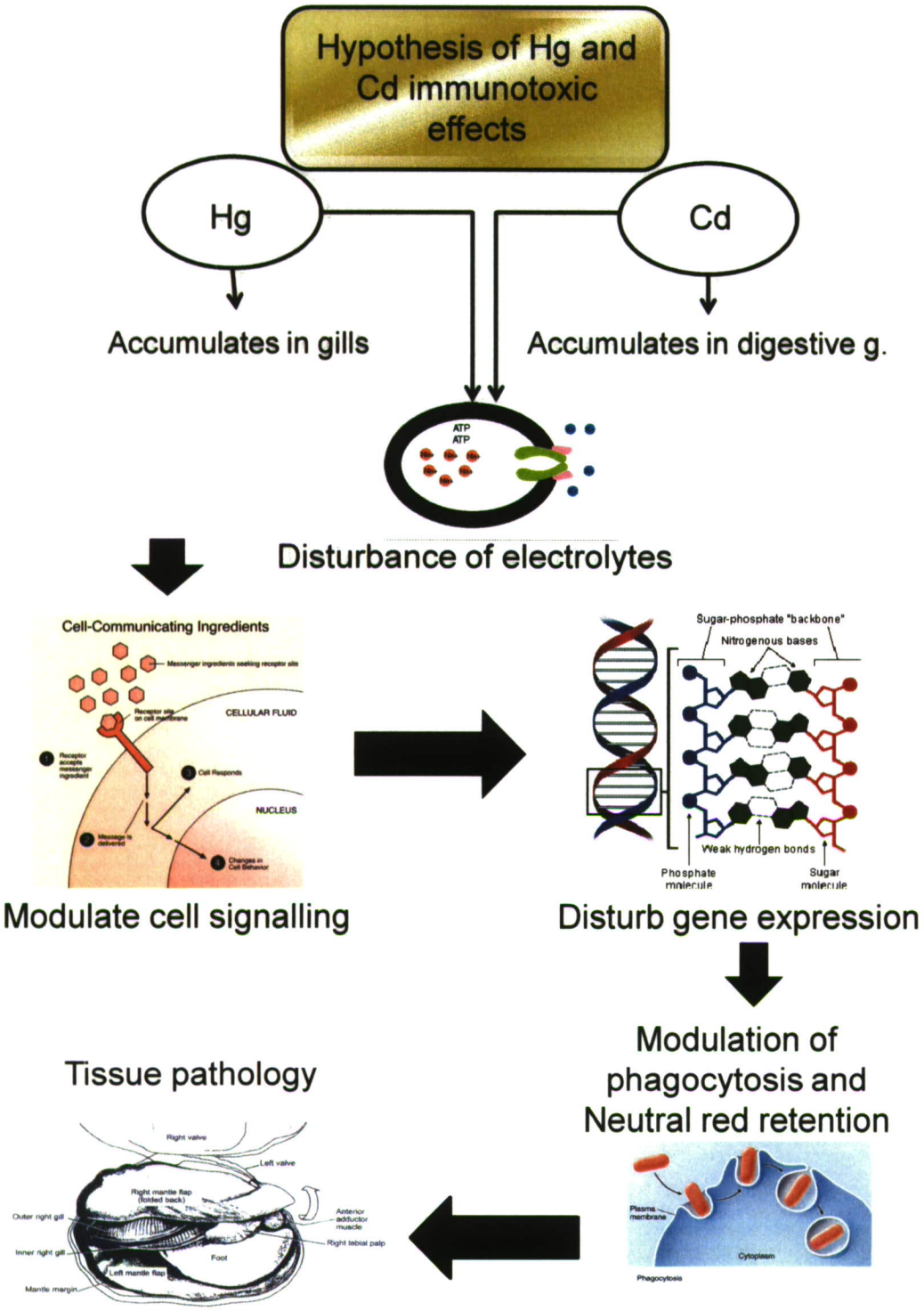

Fig. 1.2 Schematic diagram showing Hg and Cd hypothesised pathways inside the mussels' body cells

1.8 Aim of the work

This study aimed to evaluate the immunological risk caused by toxic metal exposure in bivalves. The study uses the blue mussel *Mytilus edulis* as a model organism, and used mercury and cadmium as examples of well known toxic metals that may also be immunotoxic. Hg and Cd were chosen and tested on mussels at different concentrations because they are emitted form by industry activities, and Hg has been recorded in many bivalve molluscs farming areas in high concentrations (Cossa, 1990 and Gagnaire et al., 2004 for Hg; Coles et al., 1995 for Cd).

The specific objectives:

1- To study *in vivo* immunotoxic effects of Hg to the bivalve, *M. edulis*. The hypothesis was that Hg exposure was immunotoxic to *M. edulis*. Another hypothesis was put forward that the influence of LPS pre-treatment on the response to Hg exposure would be exacerbate the effect on immune system of the mussels. So, the combined effects of LPS + Hg will be greater than Hg alone (see Chapter 3 for details).

2- To conduct an *in vivo* study on the immunotoxic effects of Cd concentration and time on *M. edulis*. LPS injection prior to Cd exposure was expected to protect the mussels from the effects of Cd by priming the immune system, or alternatively, make the organism more sensitive and exacerbate the immunotoxic effects of Cd (see Chapter 4 for details).

3- To investigate whether pre-exposure history of metal pollution will affect mussels' biological functions such as immunity, physiology and tissue pathology. In this field work, mussels were collected from a naturally polluted site and a clean site (see Chapter 5 for details).

4- To investigate whether or not the effects of sites (polluted or clean) will alter the mussels' response to subsequent Cd exposure. To achieve that, an *in vivo* exposure

of mussels from clean and polluted sites to Cd was done and immune, physiological and hitopathological parameters were measured (Chapter 6).

5. Because gene expression is now widely used as a biomarker for environmental pollution of metal toxicity, genomic techniques were applied to mussels and RT-PCR technique was chosen for quantitative gene expression, especially *mt10* gene (metal binding - protein and detoxification). *mt10* gene expression was measured in mussels' digestive gland to evaluate the effect of exposure history of pollution on the subsequent exposure to Cd (Chapter 7).

Chapter 2

Materials and methods

Materials and methods

The general methodology aimed to establish protocols for an aqueous exposure to mercuric chloride and cadmium chloride in the marine bivalve *M. edulis*, and then to examine sub-lethal effects with a focus on immunotoxicity. End points therefore include measurements relevant to the immune function of haemocyte including phagocytosis, neutral red uptake (NRU), cytotoxicity and haemocytes count assays. Biochemical measurements were made to assess oxidative stress (e.g. superoxide dismutase) and disturbances to osmoregulation (e.g., haemolymph electrolytes Na, K) and glucose. General injury to target organs as well as potential inflammation in the tissues was also assessed by detailed histological examinations of the internal organs. All the chemicals and reagents used, except where cited, were obtained from the Sigma Chemical Company (Poole, Dorset, UK). This laboratory phase enabled some bench marking of the responses of mussels to single exposures of known metals. Experimental work then moved into the field. The field work aimed to compare the health status of mussels (*Mytilus edulis*) at reference and polluted sites. The general approach included confirming environmental contamination by measuring a suite of metals in the sediment, water and mussels at the time of collection, and assessing immunological parameters and histology as above for the laboratory studies. Then a series of exposures were conducted on the field-collected animals to assess how animals with different exposure histories from the different field sites responded to a well defined cadmium exposure in the laboratory. This also involved the development of some gene expression protocols for use with *M. edulis*.

2.1 Collection of mussels for laboratory experiments

Mussels were collected at low tide from the shore at a clean reference site (Port Quin, North Cornwall, UK, grid reference SW971806, Fig. 2.1). Mussels were collected in the spring from March to May 2007 and from March to July in 2008. One hundred *M. edulis* (mean ± S.E.M., shell length, 4.5 ± 0.08 cm; fresh weight of whole mussel, 10.2 ± 0.6 g) were carefully collected to avoid damaging the animals, and immediately transferred to a cool box containing aerated seawater. Animals were returned to the laboratory within 3 h. All mussels were kept for one month (aerated filtered seawater, semi-static system with routine water changes) to ensure depuration of any food and particulate matter from the field and to adapt to the laboratory conditions. Stock animals were fed twice a week using a microalgae mix (Reed Mariculture Inc, USA), but were not fed during the experiments.

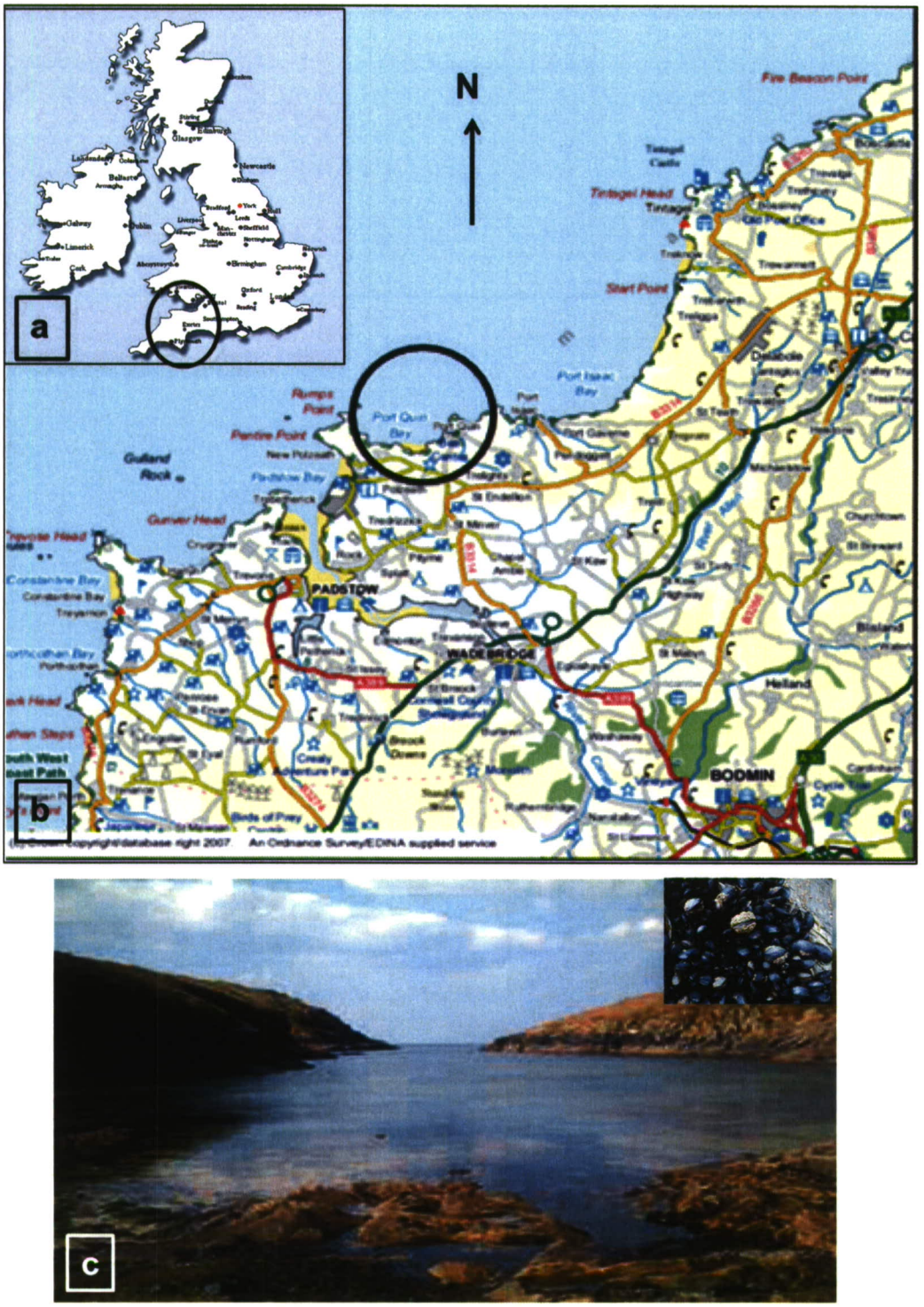

Fig. 2.1 Port Quin site
a: United Kingdom, Cornwall and Plymouth (circle)
b: Port Quin (circle)
c: Port Quin sampling site

2.2 Experimental design

The experiment was designed as described as shown in each relevant chapter. Seawater quality was confirmed by measured % dissolved oxygen, pH, total ammonia (mg l^{-1}), temperature (°C) and salinity (parts per thousand) in each of the experimental tanks using Multi 340i/SET (Germany) on sampling days the photoperiod was 12 light: 12 dark.

2.3 Haemolymph chemistry, haemocyte counts, and tissue collection

After collecting animals from the appropriate treatment, the shell was opened slightly to enable the insertion of a needle (21 gauge) and haemolymph (200 - 1000 µl) was collected from the posterior adductor muscle. Haemolymph that was intended for use in the phagocytosis and neutral red uptake assays (see below) was placed immediately into an Eppendorf tube containing an equal volume of ice cold physiological saline (in g l^{-1}; HEPES, 4.77; NaCl, 25.48; $MgSO_4$, 13.06; KCl, 0.75; $CaCl_2$, 1.47, adjust to pH 7.36) to prevent clumping/aggregation of the cells, and held on ice while other samples were collected (< 30 min to collect a batch of blood samples). Whole haemolymph samples for Hg/Cd analysis were stored in Eppendorf tubes at -80 °C until analysed. In other haemolymph samples, the cells were allowed to settle gravimetrically and the "serum" fraction was analysed for glucose concentration (see below). As well Na and K content, were measured in haemolymph (see below). Cell counts were also performed immediately using 50 µl of whole haemolymph (using a Neubauer haemocytometer). After blood sampling, the shell was fully opened and drained of any seawater, and the tissues were dissected (posterior adductor muscle, digestive gland, gills and gonads) with clean instruments, rinsed again and blotted to remove any excess deionised water for metal analysis. Tissues were processed for histology as described below.

2.4 Metal analysis

Tissue samples for metal analysis and electrolytes (Na, K and Ca) were prepared according to Federici et al. (2007) with minor modifications. Briefly, about 0.05 g of fresh tissue (haemolymph, adductor muscle, digestive gland, gills or gonads) was oven dried to a constant weight, and then digested in1 ml of concentrated Aristar HNO_3 for 2 h at 70 °C. Samples were cooled, and then diluted to a final volume of 5 ml using ultrapure deioinized water. Total Hg, Cd, Pb, Cu, Na, K, Ca, Fe and Zn were measured using inductively coupled plasma-mass spectroscopy (ICP-MS, Fisons Instruments, and VG Plasma Quad PQ2 ICP-MS) against certified Hg/Cd standards containing 1 % thallium as an internal standard. The standards also contained 2 % nitric acid so that they were matrix matched to the samples. Na^+, K^+, Ca^+ were mentioned as ions when relevant.

2.5 Immunological assays

Three standard assays were performed on haemocytes: a neutral red uptake assay, a phagocytosis assay, and a cytotoxicity assay.

2.5.1 Neutral red assay

This assay was performed according to Babich and Borenfreund (1992). The assay measures neutral red uptake by the haemocytes, and has been used as a general indicator of cell health (lysosomal membrane stability) or viability. Whole haemolymph (50 µl) was placed in triplicate wells of a flat bottomed microtitre plate (Sero-Wel, 96) that had been pre-coated with 10 % (v/v) poly-L-lysine solution to improve cell adhesion. The plates were covered and incubated at 4°C using a refrigerator for 50 mins to allow cell adhesion.

Then the excess haemolymph plasma containing non-adherent hemolytic cells is carefully pipetted out from the wells (using a multipipettor, thereby ensuring that adherent cells are not disturbed). Then 200 µl of 0.004% (w/v) neutral red solution was added to each well and the cells incubated at room temperature in the dark (NR is light sensitive) for 3 h to allow cells to take up the neutral red. After 3 h the excess neutral red was carefully poured away, and the cells washed once very gently with physiological saline. In order to release the neutral red, cells were lysed with 200 µl of acidified ethanol (1% acetic acid and 50% ethanol in D. W.) which was added to each well and then the plate was shaken gently for 30 seconds to ensure dispersion/dissolution of the released neutral red. Plates were read at 550 nm in an OPTI max tunable plate reader (Molecular Devices), using Softpro Max (V.2.4.1) software.

About 10 µl of the final lysate in wells was kept for protein assay following a recognised method (Bradford, 1976). Neutral red uptake was expressed per mg of cell protein using the following equation:

$$\text{NR Optical density/mg protein} = \frac{\text{Neutral red absorbance of sample at 550 nm}}{\text{Protein concentration}}$$

2.5.2 Phagocytosis assay

The phagocytosis assay was performed according to Pipe et al. (1995a). The phagocytosis assay measures the ability of haemocytes to engulf foreign materials (neutral red-stained zymosan particles). At room temperature, 50 µl of haemolymph was carefully drawn from each of the cooled Eppendorf tubes above, and placed into wells of a flat bottomed microplate (Sero-Wel, 96) that had been pre-coated with poly-L-lysine. The plate was then agitated on a plate shaker (MS1 minishaker) at 400 rpm for 60 secs to mix the haemocytes. The plate was then covered (with plate cover) and

haemocytes were allowed to rest on to the bottom of a microplate in a refrigerator at 4°C for 50 mins to form monolayer (cells adhere to the well). Then 100µl of Baker's formal calcium (2% NaCl, 1% $C_4H_6CaO_4$ and 4% formaldehyde) was added to negative controls (to kill the cells) for 10 mins at room temperature. Then 50 µl of zymosan/neutral red solution (see dyed zymosan suspension preparation below) was added to the test wells and the cells incubated for a further 30 mins at 10 °C to allow the cells to phagocytose the zymosan particles. Then 100 µl of Baker's Formal calcium was added to all wells to stop the reaction, and incubated for 10 mins at room temperature. The plate was then spun at 140 *g* (Centurion Scientific Ltd, 1040) for 5 mins to separate the supernatant containing non-phagocytosed particles. The supernatant was then removed carefully using a pipette (about 100 µl). The remaining cells were then carefully washed in 100 µl physiological saline, the plate was spun again, and the physiological saline was carefully discarded so as not to disturb the cells. Cells were washed until the wells with the negative controls which containing dead haemocytes were clear (about three times). Then the plate was spun again, after adding 100 µl of a dyed zymosan standards series to some wells to produce a standard curve (see Table 2.1 for standards).

Dyed zymosan suspension preparation

1 % neutral red stock was prepared by dissolving 0.1g neutral red powder in 10 ml of distilled water. 3 ml of 1 % neutral red was added to 1 g of zymosan and dissolved with distilled water in a test tube. The tube was shaken thoroughly to make sure every particle of zymozan is exposed to neutral red staining. The mix was allowed to stand for 15 mins. Seven ml of distilled water was added to the suspension and mixed thoroughly. The mixture was fixed by heat in a water bath for 15 mins. The mixture was left to cool and then centrifuged at 250 *g* for 5 mins. The supernatant was removed and the pellet

resuspended in PBS. The last step was repeated until the supernatant became clear. The dyed zymosan suspension was resuspended in physiological saline at a concentration of 5 x 10^8 particles per ml as a stock suspension (A) (Table 2.1). Finally the suspension were stored at 4°C until used.

Table 2.1 Zymozan standards preparation

Concentrations/10^7 particle/ml	Dyed zymosan suspension (stock A)	Physiological saline
50	1000 µl of stock A	--------
25	500 µl of stock A	500 µl
12.5 (stock B)	500 µl of stock A	1500 µl
6.25	500 µl of stock B	500 µl
3.125	250 µl of stock B	750 µl
1.56	125 µl of stock B	875 µl

The dye particles in each well were solubilised by the addition of 100 µl of acidified ethanol (1% acetic acid + 50% ethanol, up to 100 ml D.W.) to all wells. To ensure dissolution of the dye particles, the plates were left for 10 mins then shaken gently at 200 rpm for 1min (MS1 minishaker). Samples and standards were read immediately at 550 nm (OPTI max tunable microplate reader). An example calibration curve is shown in Fig. (2.2). About 10 µl was used for protein assay (see below).

The number of zymosan particles phagoctosed was calculated using the following equation and expressed per mg of cell protein.

No. of zymosan Particles phagocytosed 10^7/mg protein =

$$\frac{\text{No.of zymosan particles phagocytosed by sample}}{\text{Protein (mg)}}$$

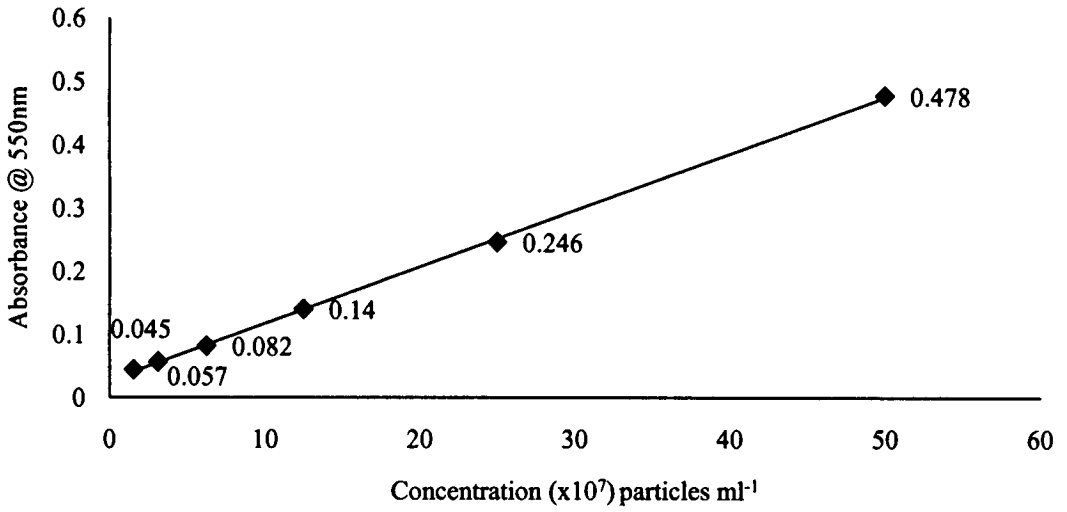

Fig. 2.2 Standard curve of zymozan particles. Zymozan particle concentrations (1.65 to 50 x10^7 particle /ml) were prepared in D.W. and data points are the mean of triplicates. The r^2 value for a linear fit was typically 0.999. y = 0.00089x + 0.0279

2.5.3 Erythrocyte cytotoxicity assay

A cytotoxicity assay was performed to determine the ability of haemocytes at killing foreign cells (i.e., cytotoxicity to sheep red blood cells). The assay measures the release of haemoglobin from sheep red blood cells as they are lysed by the haemocytes. This assay was performed according to Raftos and Hutchinson (1995). The absorbance value of released haemoglobin into the supernatant allows a cytotoxicity value to be attached to the haemocyte population. 1 ml of sheep erythrocytes (sheep red blood cells in Alsevers, Tissue Culture Services, UK) was centrifuged (2000 *g*, 5 mins, Sigma 3K30). The red blood cells were washed in PBS to remove any cells that had died, gently centrifuged to make a soft pellet (300 *g*) without damaging the washed red cells, and the supernatant was discarded. The cells were resuspended in 1ml of PBS after each wash. After the final centrifugation, the supernatant was discarded and the washed red blood cells were resuspended to a cell pack volume of 0.125 ml in 1ml of a calcium containing Tris buffered saline (TBS + Ca: 10 mM Tris - HCl, 150 mM NaCl, 10 mM $CaCl_2$ and made up in 100 ml D.W., adjusted to pH 7.4). Further dilution of 1ml of cell suspension in 15 ml TBS + Ca was done to approach specific number of red blood cells.

Then 100 µl of haemocyte (adjusted to a density of $2x10^6$ cell ml^{-1} in the Eppendorf) suspension in physiological saline was placed in triplicate to a round-well microplate (Sero-Wel, 96). For controls, 100 µl TBS + Ca was added for spontaneous haemolysis of cells and release of haemoglobin; and 100 µl of TBS + Ca with 2 µl triton X - 100 to lyse all cells and give maximum release of haemoglobin. Then 100 µl of the erythrocyte suspension was added to each test well and incubated for 1 h at room temperature to lyse the sheep red blood cells. After incubation, the microplate was spun (2000 *g*, 10 minutes) to get rid of cell debris. Then 100 µl of the supernatant (which contained haemoglobin in solution from the lysed sheep cells) was transferred to a flat-bottomed microplate. Absorbance was read at 405 nm on an OPTI max tunable plate reader. The % cytotoxicity was calculated from the following equation:

% cytotoxicity =

$$\frac{\text{Absorbance of the sample - absorbance of the spontaneous control}}{\text{Absorbance of maximum control - absorbance of spontaneous control}} \times 100$$

2.6 Superoxide dismutase (SOD)

Treatment with lipopolysaccharide (LPS) may stimulate superoxide production by haemocytes as the immune system is activated. I therefore included measurements of superoxide dismutase (SOD) in the haemolymph (extracellular SOD) and in the haemocytes (intracellular SOD). The superoxide dismutase assay was conducted according to McCord and Fridovich (1969) to measure the oxidative stress response of the haemolmph and isolated haemocytes in some experiments with an LPS challenge and/or Hg/Cd exposure.

Haemolymph samples were spun at 10,000 g for 5 mins, the supernatant was collected into a clean eppendorf tube to measure the "cell free" or "extracellular SOD" specific activity. Then 40 µl of potassium phosphate buffer (50 mM l^{-1} Potassium

phosphate pH 7.8 treated with Chelex-100 in dialysis tubing) was added to the pellet, frozen at -80 °C, and then thawed to lyse the cells, vortexed, spun at 10,000 *g* for 5 mins and supernatant was used for intracellular SOD.

The assay mix consists of three reagents (xanthine, xanthine oxidase and cytochrome *c*) dissolved in potassium phosphate buffer, stored at room temperature during use and at 4 °C between uses. Xanthine (0.5 mM l^{-1} in 20 ml D.D.W.) was prepared fresh each day and stored at room temperature during use. Xanthine oxidase (5 µl of Sigma X4500 diluted with 495 µl of potassium phosphate buffer), prepared fresh each day, stored on ice during use, and used within a few hours. Cytochrome *c* (25 mg ml^{-1}) prepared in phosphate buffer and stored on ice during use and at -20 °C between uses. Assay mix is prepared from 8.85 ml potassium phosphate buffer; 1 ml xanthine; 50 µl cytochrome *c*. High concentration of superoxide dismutase was prepared fresh each day by diluting 10 µl of (bovine erythrocytes) Sigma S8409 with 90 µl buffer and stored on ice during use.

10 ml of assay mix was prepared just before use. This is sufficient for at least 32 wells. 10 µl of samples, two sets of blank (buffer) and one for high concentration of SOD was pipetted out in triplicate into each well. 100 µl xanthine oxidase solution was added immediately before use to assay mix, wells were loaded and readings started within 1 minute of this addition. 250 µl of assay mix were pipetted into each well using a multichannel pipette, and mixed carefully with the pipette. The plate was immediately transferred to the plate reader, automixed for 5 sec and the increase in absorbance monitored at 550 nm kinetic Program for at least 5 min. All data expressed as units ml^{-1} as a half rate of cytochrome *c* reduction ± S.E.M.

The assay is a kinetic rather than end point method, and the specific activity is calculated from the maximum rate of change (initial rate) in absorbance of the sample at 550 nm (A_{550}). The raw data on the initial rate of increase of A_{550} were exported to

Excel and then to Sigma Plot for processing. Briefly, time courses were fitted with an exponential rise to maximum (3 parameter), and the initial rate calculated from the fitted parameters. The rate of increase of A_{550} in the absence of SOD activity should be around 2 - 3 × 10^{-4} s^{-1}. All rates were corrected for background by subtracting the SOD-insensitive rate. Typically the rate of SOD is between 5 and 10 % of the rate in the absence of SOD.

2.7 Determination of haemolymph glucose

Haemolymph glucose determination was performed according to Barham and Trinder (1972). The glucose reagent was prepared in 0.1 M phosphate buffer (pH 7). Then 10 µl of each sample (standard, test sample, or blank) was added in triplicate to 3 ml of the glucose reagent (0.5 mmol l^{-1} 4-aminoantipyrine; 20 mmol l^{-1} p-hydroxybenzene sulphonate; 15,000 U l^{-1} glucose oxidase (from *Asperigillus niger*); 10,000 U l^{-1} horseradish peroxidise) or 5 µl to 1ml of glucose reagent and was incubated in the water bath at 37°C for 10 mins using stop watch. Samples were staggered to ensure each sample had 10 min incubation. The spectrophotometer was blanked using 1 ml of the glucose reagent. Glucose standards of 1, 5, 10 and 20 mmol l^{-1} were used (Fig. 2.3) and read in a spectrophotometer (Jenway, 6300) at 505 nm at the same time intervals after the incubation. The glucose levels in the unknown samples were calculated from the calibration curve.

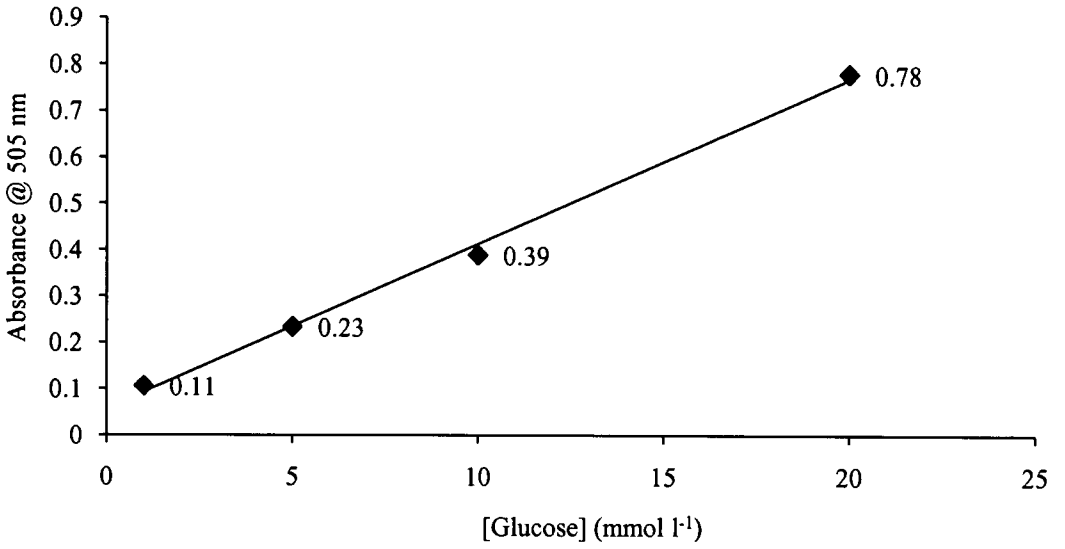

Fig. 2.3 An example standard curve for the glucose assay. Glucose concentrations (0 to 20 mmol l^{-1}) were prepared in D.W. and data points are the mean of triplicates. The value r^2 for a linear fit was 0.996. y = 0.035x + 0.057.

2.8 Determination of Na and K concentrations in haemolymph

Na and K concentrations were measured in whole haemolymph. Samples were analysed by flame atomic absorption spectrophotometry (F-AAS) using a Corning 480 flame photometer. Samples were analysed after a ten times dilution, against ready prepared glass vials of 140 mmol/l Na and 5 mmol/l K standards (Corning). The flame photometer was switched on, and aspirated with deionised water for 3 minutes, before setting the zero. Then the standards were read to calibrate the instrument, before running the test samples. In order to prevent instrument drift and ensure good calibration, the instrument was reset to zero and re-calibrated with the standards every 10 samples to check the calibration.

2.9 Histology

Histological examinations were carried out at the end of each exposure. Mussels were removed from their shells (6 animals/treatment for laboratory studies, 9 animals/treatment for field work), and whole gill lamellae, half the posterior adductor muscle and a portion of digestive gland were carefully collected and fixed in a formaldehyde solution (9 g NaCl, 100 ml of 40 % formaldehyde, made to 1 l with distilled water, adjusted to pH 7.4) for 1 week (Fig. 2.4). Samples were then dehydrated through a series of alcohols to remove excess water (70 % for 24 h, 90 % industrial methylated spirit, IMS, for 2 h, and 100 % IMS for 2 h), cleared in three changes of xylene (1 h, 30 mins and 30 mins, respectively) to remove alcohol and to leave the tissue ready for paraffin infiltration. Tissues were transferred to the paraffin oven (58 – 60 °C) for 60 - 120 mins to ensure the tissue was completely permeated with paraffin and manually set in paraffin wax blocks, which were then left to harden for 3 h. Transverse sections (5-8 μm thickness) were cut and stained with Haematoxylin and Eosin (Mayer's H and E) following standard protocol with some modifications of staining times to obtain the best results. Staining time was adjusted as follows: slides were cleared in 3 Xylene changes for 2 mins each. They were pre-dehydrated in 2 changes of Absolute Alcohol; 90, 70, and 50 % for 2 mins each. Slides were stained in Haematoxylin for 60 mins. Staining was followed by a good wash with tap water. Then the slides were blued in $LiCo_3$ 5 rinses, differentiated in Acid Alcohol (2 rinses) and blued again in $LiCo_3$ 5 rinses. Followed by a good wash with D. W., and then stained with Eosin for 1 min. After staining, a good wash with D. W. was done. Dehydration with descending Alcohol series for 2 mins each was applied. Clearing in 3 changes of Xylene for 2 mins each was done. Then the slides were covered with clean coverslips making sure that there was no air bubbles trapped underneath them. Tissues from control mussels and treated animals were processed together in batches for histology to

eliminate artefacts between treatments. Slides from each animal were examined by light microscopy using an Olympus Vanox-T microscope, and photographed (Olympus camedia C-2020 Z) at magnifications of x 400 and x 1000. The thickness of the tubule walls in the digestive gland was measured manually on collected images and a mean for each section derived by randomly counting at least 10 tubules. The percentages of tubules with injuries (total injury as the presence of any type of pathology and tubules with necrosis), in each digestive gland were counted manually from a randomly selected area on a section from each animal. The same method was used in gill and gonad sections. Tissue inflammation was categorized as -, absent inflammation; +, rare inflammation; ++, moderate inflammation, and +++, frequent inflammation. The proportions of tubule area in the digestive gland or egg/ testicular follicles of female/male gonads section of each animal were calculated using the point counting method of Weibel et al. (1966). Where the fractional volume (area) $V_i = P_i / P_T$; and P_i is the number of points counted, P_T is the total number of points on the counting grid.

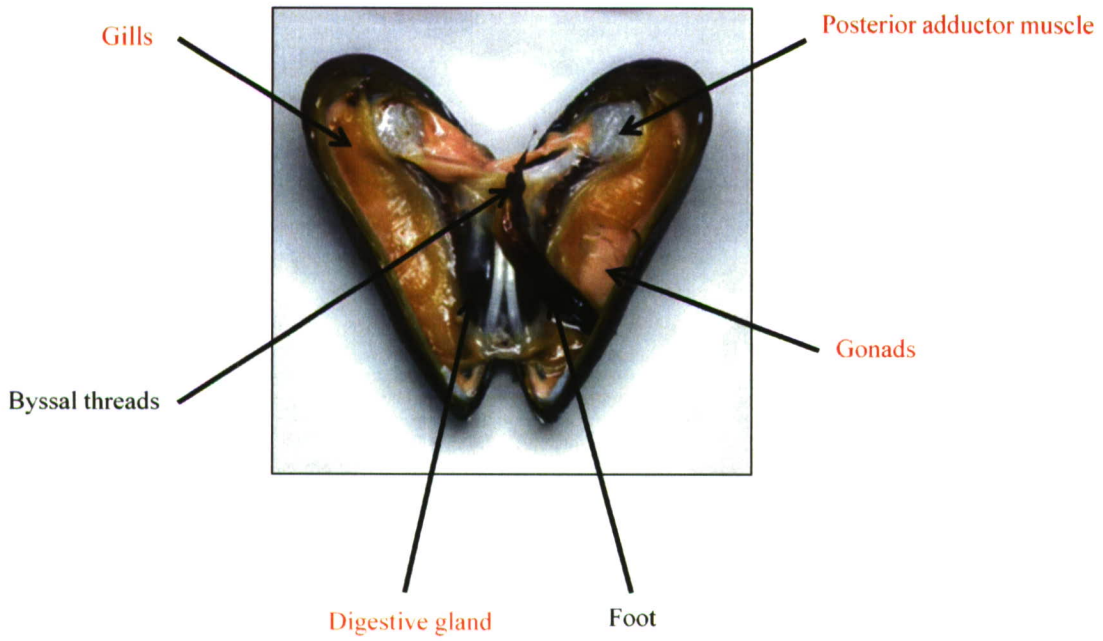

Fig. 2.4 Dissected *M. edulis* showing some of the internal organs and selected organs (red) for histology

2.10 Statistical analysis

All data were analysed using Statgraphics 5.1 Plus software, using a rejection level of $P = 0.05$. After some initial descriptive statistics the data were analysed. No tank effects were observed within triplicates for any of the laboratory experiments, so the data were pooled by treatment for statistical analysis. Data were analysed using two-way ANOVA looking for combined treatment and time effects. When time × treatment interaction was significant, one-way ANOVA was applied to obtain additional resolution in some data and to locate specific treatment and time effects within ANOVAs. Bartlett's test was performed for variance check. Where ANOVA could not be applied, a non-parametric ranking test was used (Kruskal Wallis test).

Chapter 3

Tissue injury and cellular immune responses to mercuric chloride exposure in the common mussel *Mytilus edulis*: modulation by lipopolysaccharide

Tissue injury and cellular immune responses to mercuric chloride exposure in the common mussel *Mytilus edulis*: modulation by lipopolysaccharide

Abstract

Little is known about the immunotoxic effects of sublethal mercury exposure in bivalves, and whether or not stimulation of the immune system exacerbates mercury toxicity. The mussel, *Mytilus edulis*, was exposed to 50 or 500 μg l^{-1} total Hg for up to 11 days compared to no added Hg controls to assess immune functions of haemocytes, haematology, biochemistry and organ histology. Selected experiments were then repeated in mussels injected with lipopolysaccharides (LPS) to stimulate the immune system prior to Hg exposure. Mercury exposure alone caused accumulation of Hg mainly in gills and digestive gland, and to a lesser extent in the adductor muscle and haemolymph. This general pattern of Hg accumulation occurred during Hg + LPS experiments, although tissue Hg levels were higher than Hg exposure without LPS. Mercury exposure alone caused a transient modulated of phagocytosis, an increase in neutral red uptake by haemocytes, and decreased cytotoxicity to haemocytes. Changes in haemolymph Na, K and glucose levels were negligible during Hg exposure alone compared to controls. However, histopathological examination showed tissues injuries consistent with inflammation in the gills, digestive gland and adductor muscle during Hg exposure. LPS injection had a significant effect (Kruskal Wallis, $P = 0.02$) on circulating haemocytes numbers at the end of the experiment compared to saline injected controls and Hg + LPS (mean ± S.E.M., $n = 6$, cells ml^{-1} x 10^6); control, 2.1 ± 0.08; LPS treated, 3.18 ± 0.36, and LPS + Hg, 2.1 ± 0.2. The Hg + LPS treatment also caused a transient decline in superoxide dismutase activity in haemocytes (85 % decline, Kruskal Wallis, $P = 0.006$) compared to controls. Target tissue inflammation and pathology was greatly increased by the effect of Hg exposure with an LPS injection

compared to either treatment alone. We conclude that Hg can cause inflammation in mussels *in vivo*, and that stimulation with LPS can greatly increase Hg-dependent immunotoxicity.

3.1 Introduction

The ecotoxicology and chemistry of mercury (Hg) have been extensively studied in wildlife and aquatic systems (e.g., Wolfe et al., 1998; Wang et al., 2004). Inorganic Hg is a persistent toxic substance that rapidly associates with colloid and other ultrafine materials in water and therefore presents a risk to organisms that filter the water such as bivalves. Measurement of total Hg in bivalves from contaminated ecosystems show appreciable Hg accumulation in *Mytilus edulis* (e.g., 69 to 378 ng g^{-1} dry weight (dw), Cossa and Rondeau, 1985; 6.5 μg g^{-1} dw, Szefer et al., 1999). The LC_{50} estimate for inorganic Hg in adult *M. edulis* is between 0.5-2.0 mg l^{-1} (Børseth et al., 1992); sublethal effects are therefore expected at μg l^{-1} concentrations. These sublethal effects include reductions in the scope for growth and changes in cardio-respiratory functions (Beiras and His, 1995). However, there are only a few reports of the effects of inorganic Hg on haemolymph composition (e.g., Ulevitch and Tobias, 1995), and functions in bivalves (Sauvé et al., 2002; Thiagarajan et al., 2006).

The haemolymph and haemocytes are important components of the immune system of invertebrates (for review, see Galloway and Handy, 2003), and there are concerns that pollutants in aquatic systems can cause immunosupression or other types of immunotoxicity (Galloway and Depledge, 2001). The immune system of bivalves relies on the circulating haemocytes being able to penetrate tissue in response to antigens, and the immune system has both innate and humoral components, in which haemocytes play a key role (Canesi et al., 2002). Many classes of environmental pollutants have been implicated in the modulation of immune function, including metals

(e.g., Cossa and Rondeau, 1985). Immunotoxicity from Hg exposure has been documented in mammals and fish (Sweet and Zelikoff, 2001), but there are few reports on invertebrate species (Boisson et al., 1998; Sauvé et al., 2002; Duchemin et al., 2008). Several studies have demonstrated toxic effects of metals to haemocytes from bivalves *in vitro*, but findings are conflicting, with reports of suppression and stimulation of phagocytosis in haemocytes (Brousseau et al., 1999; Gagnaire et al., 2004).

Lipopolysaccharides (LPS) are important components of the external membranes of Gram negative bacteria and are known to stimulate immune responses in eukaryotes by several methods, including the activation of signal transduction pathways in macrophages (Ulevitch and Tobias, 1995). LPS at high concentrations can produce massive over-stimulation of the immune system and acute hypersensitivity reactions (Freudenberg et al., 2008), and these effects are also associated with the LPS components of algal blooms in aquatic systems (e.g., in exposed fish, Best et al., 2002). However, at low μg concentrations, LPS can be used as an investigative tool to produce a controlled stimulation of the immune system. LPS has been used to investigate the expression of protein kinases in haemocytes from marine mussels (*M. galloprovincialis*, Coa et al., 2004) and the effects of temperature on immune responses using haemocytes from *M. edulis* (Hernorth, 2003a).

The overall aim of this study was to add to the literature on the immunotoxic effects of Hg to bivalves, by performing an *in vivo* study with *M. edulis*. Two trials were conducted within the experimental design. The first series tested the hypothesis that Hg exposure was immunotoxic to *M. edulis*. In this experiment, mussels were exposed to Hg alone, with measurements of total Hg accumulation in tissues, immune responses of haemocytes, haemolymph chemistry, and organ histology. In the second series of trials, the influence of an LPS pre-treatment on the response to Hg exposure was examined, and compared to the Hg alone treatment in the previous trial. The working hypothesis

was that LPS pre-treatment would adversely sensitize the immune system of the mussels, so that the combined effects of LPS + Hg were greater than either treatment alone.

3.2 Materials and methods

3.2.1 Experimental design

The experiment was split into two consecutive trials for logistic reasons, and this was unavoidable and absolutely essential so that all the haemocyte assays could be performed fresh immediately at each time point on the day of sampling. The first trial was conducted to explore the toxic effects of 50 and 500 $\mu g\ l^{-1}$ total Hg added as $HgCl_2$ to mussels *in vivo* over 8 days compared to a no-added Hg controls. This sub-lethal concentration was chosen following range finding experiments using Hg concentrations of 0-500 $\mu g\ l^{-1}$, and was selected on the basis of good haemolymph chemistry (to exclude osmoregulatory stress as confounding factor in haemocyte function) and the absence of mortality during pilot studies (data not shown, 500 $\mu g\ l^{-1}$ was lethal after 6 days). Samples were collected on days zero, 4, and 8 for phagocytosis, neutral red uptake, and cytotoxicity assays to assess the immune functions of haemocytes from the mussels. Haemolymph Na, K, glucose levels were also measured. As an additional, histology was conducted on animals where the exposure was left to continue for a further 3 days (day 11), on gills, digestive gland and adductor muscle. A total of 90 animals were randomly allocated to the tanks (10 animals/tank, in triplicate = 30 animals/treatment), and all tanks were acid-washed (10 % HNO_3) and rinsed in clean seawater prior to use. Each tank (L: 30.3 cm, W: 10 cm and H: 20.5 cm) contained 10 litres of filtered sea water maintained at a constant temperature (16 ± 1 °C). Mercury stock solutions were prepared by dissolving 0.077 g and 0.77 g of $HgCl_2$ each in 500 ml of acidified Millipore water respectively, and 5 ml of one of these stock solutions was

added to the appropriate tank to give nominal total Hg concentrations of 50 or 500 μg l^{-1}, respectively. A semi-static exposure method was used. Tanks were re-dosed every 24 h with 5 ml of the stock solution, after a 100 % water change (performed by siphoning). The exposure was confirmed by measured total Hg concentrations in the tanks (see method below) on sampling days and was (mean ± S.E.M., n = 24): 46.7 ± 3.3 and 513 ± 10.4 μg l^{-1} for the 50 and 500 μg l^{-1} treatments respectively. Background Hg levels in the control filtered sea water was 1.48 ± 0.1 μg l^{-1} or less. The stock seawater quality was: % dissolved oxygen (95.2 ± 0.2), pH (7.97 ± 0.01), total ammonia (0.05 ± 0.01 mg l^{-1}) and salinity (31.6 parts per thousand). Temperature, dissolved oxygen, ammonia and pH were measured every day for husbandry purposes and remained within the limits outlined above.

The second trial aimed to explore the effects of LPS (from *E. coli*, 055: B5) with and without Hg exposure. The 50 μg l^{-1} Hg concentration was also used for this trial. The measured end points and number of mussels/treatment used were identical to the first series (triplicate design, 10 animals/tank, 30 animals per treatment, 90 animals in total), except that the experiment was shorter with sampling at 0, 24 h, 4 and 8 days, with histology at the end of the exposure (day 8). In addition, superoxide dismutase activity in the haemocytes was also measured (see below). Mussels were divided into 3 treatments; sham injected saline control (no LPS or Hg), with LPS injected in the posterior adductor muscle (LPS alone, no Hg), and LPS injected followed by Hg exposure (LPS + Hg exposure). The LPS stock (obtained from Sigma) contained 2 mg ml^{-1} LPS and each animal was injected with either 50 μl of saline (control), or the LPS to give a total LPS dose of 100 μg per animal. This dose was selected after reviewing doses used in previous experiments with *M. edulis* (Smith et al., 2000). Sham injections were done with 50 μl of phosphate buffered saline (PBS tablets, Sigma, P-4417).

Haemolymph chemistry, haemocyte counts, tissue collection, metal analysis, immunological assays like neutral red uptake, phagocytosis and cytotoxicity, superoxide dismutase and histology assays were done as shown in materials and methods, Chapter 2.

3.2.2 Statistical analysis

All data are expressed as means ± S.E.M. for n = 6 - 9 mussels per treatment. In the tissue Hg analysis, where values were at or below the detection limit, the detection limit value was used in statistical analysis. See Chapter 2 for more details.

3.3 Results

3.3.1 Hg accumulation, tissue electrolytes and immunological responses to Hg exposure

The first experiment explored the effects of exposure to either 50 or 500 μg l^{-1} Hg. There were no mortalities in the controls and a cumulative mortality of only 3.3 % over the entire experiment for the 50 μg l^{-1} treatment. However the 500 μg l^{-1} Hg treatment caused some lethal toxicity in the first experiment, with a cumulative mortality of 66 %. Most of these mortalities occurred on days 6, 7, and 8 of exposure (13, 2, and 4 mortalities or 43, 6 and 13 % mortality respectively on each of these days).

Mercury exposure was also confirmed by clear evidence of Hg accumulation in the tissues (Table 3.1). Two-way ANOVA revealed significant time and treatment effects (P-values ≤ 0.001 and 0.00001, respectively). Analysis of mercury concentration in the adductor muscle, digestive gland and gills demonstrated highly significant increases in tissue levels of Hg in the Hg treatments compared to the control, with Hg concentrations being highest in the gill > digestive gland > posterior adductor muscle

(Table 3.1). The treatment-effect showed highly significant differences from the controls (Kruskal Wallis, $P = 0.01$ or much less) for all tissues at day 4 onwards (Table 3.1). In some samples from the 500 μg l^{-1} Hg exposure, only a few animals survived at day 8 (not enough for statistical analysis of tissue Hg), but even these samples showed Hg levels that were orders of magnitude higher than the controls (Table 3.1).

Similar to the tissues, haemolymph showed elevation of Hg levels in a time and treatment-dependent manner (two-way ANOVA, $P = 0.0$, Table 3.1). Hg levels were significantly higher than the controls by day 4 in both Hg treatments, and remained elevated during the experiment compared to controls which remained at or below detection limit. A separate analysis of time and treatment effects also showed highly significant differences (Kruskal Wallis, $P < 0.01$).

Table 3.1 Mercury concentrations in gills, digestive gland, adductor muscle and haemolymph of *M. edulis* exposed to 50 and 500 μg l^{-1} Hg as $HgCl_2$

Tissue	Hg Treatments (μg l^{-1})	Time (days) 4	8	11
Gills	Control	< 4.0	6.2 ± 0.4	13.8 ± 2.3
	50	121.8 ± 20.8	367.2 ± 40.4*#	439.5 ± 71.1*#
	500	210.9 ± 62.8*	259.5	
Digestive gland	Control	< 4.0	< 4.0	4.8 ± 0.9
	50	19.6 ± 4.7*	90.5 ± 4.7*#	112.0 ± 24.4*#
	500	8.3 ± 0.8	24.1	
Adductor muscle	Control	< 4.0	< 4.0	< 4.0
	50	< 4.0	5.6 ± 1.1*#	7.7 ± 0.4*#
	500	8.4 ± 2.4*	6.6	
Haemolymph	Control	< 8.0	< 8.0	8.5 ± 1.9
	50	26.7 ± 13.6	42.0 ± 7.6 *#	51.3 ± 12.1*#
	500	131.9 ± 15.3*	77.3	

Data are means ± S.E.M. μg g^{-1} dw for tissues and ng ml^{-1} for haemolymph, $n = 6 - 9$ mussels per treatment at each time point (except in the 500 μg l^{-1} Hg treated mussels on 8 day where only one mussel survived and none survived by day 11). * indicates significant difference from the respective control within each time point. # indicates a significant difference in time within treatment, $P \leq 0.05$, ANOVA or Kruskal Wallis. Note, values for initial animals (time zero) at the start of the experiment were under detection limit for tissue Hg, 4 μg g^{-1} dw and 8 μg l^{-1} for haemolymph.

Tissue (gills, digestive gland and adductor muscle) electrolytes (Na, K and Ca) were measured in on day 11. Electrolyte composition of the tissues did not change during Hg exposure (Table 3.2). No significant differences were detected between the control and treated mussels with 50 μg l^{-1} Hg in the examined organs (ANOVAs, $P > 0.05$). However, gills from Hg-treated animals showed a statistically significant decrease of Ca content compared to controls (ANOVA, $P = 0.01$, Table 3.2).

Table 3.2 Na, K and Ca concentrations in the gills, digestive gland and adductor muscle of *M. edulis* after 11 days exposure to 50 μg l^{-1} Hg as $HgCl_2$

Element (μg g^{-1} dw)	Treatment	Gills	Digestive gland	Adductor muscle
Na	control	41.1 ± 0.04	20.7 ± 0.0	16.3 ± 1.9
	50 μg/l	26.9 ± 1.8	22.2 ± 2.6	25.9 ± 7.1
K	control	14.3 ± 0.04	15.7 ± 0.0	11.7 ± 1.6
	50 μg/l	12.6 ± 0.7	15.2 ± 1.1	8.6 ± 2.7
Ca	control	11.4 ± 0.4	8.1 ± 0.0	5.7 ± 0.8
	50 μg/l	7.3 ± 0.6*	10.6 ± 2.5	16.0 ± 5.7

Data are means ± S.E.M. μg g^{-1} dry weight, $n = 6$ mussels per treatment. * indicates a significant difference from the control within each time point (ANOVA or Kruskal Wallis, $P < 0.05$).

The neutral red uptake of haemocytes assay is shown (Fig. 3.1a). Two-way ANOVA revealed individual time or treatment effects, but no combined effect (treatment × time, $P = 0.2$). Exposure of mussels to mercury, resulted in a statistically significant increase in neutral red uptake by the haemocytes of animals from the 500 μg l^{-1} treatment at 4 days (Kruskal Wallis, $P = 0.008$). However, by the end of the experiment only one animal could be assessed for neutral red due to mortality, and this animal showed a much higher retention than all other animals (Fig. 3.1a). Mussels from the 50 μg l^{-1} Hg treatment did not show increases in neutral red uptake compared to controls (no significant treatment-effect), although neutral red uptake increased over time in all treatments (Fig. 3.1a).

Mercury exposure resulted in a transient stimulation of phagocytic activity that peaked at day 4 of exposure (1.58 fold increase). These effects were lost by day 8 of the experiment, with phagocytosis in the Hg-treated group returning to control values. The control and 50 $\mu g\ l^{-1}$ treatment showed a statistically significant rise compared to the initial animals at time zero or the end of the experiment (Kruskal Wallis, $P = 0.03$ and 0.01, respectively), but the 500 $\mu g\ l^{-1}$ treatment did not (Fig. 3.1b).

The cytotoxicity assay assessed the ability of the haemocytes from the mussels to lyse sheep erythrocytes on day 11. However, no statistically significant treatment effects were observed. At day 11 of exposure, values were 88.9 ± 10.1 and 73.01 ± 2.7 %, for the control and 50 $\mu g\ l^{-1}$ treatments, respectively. Although this difference was not statistically significant due to inter-animal variability in the Hg treatment (Kruskal Wallis, $P = 0.2$), it does suggest some declining capacity for cell killing by some haemocytes. Insufficient animals survived in the 500 $\mu g\ l^{-1}$ treatment to assess the ability of their haemocytes to cause cytotoxicity at the end of the experiment.

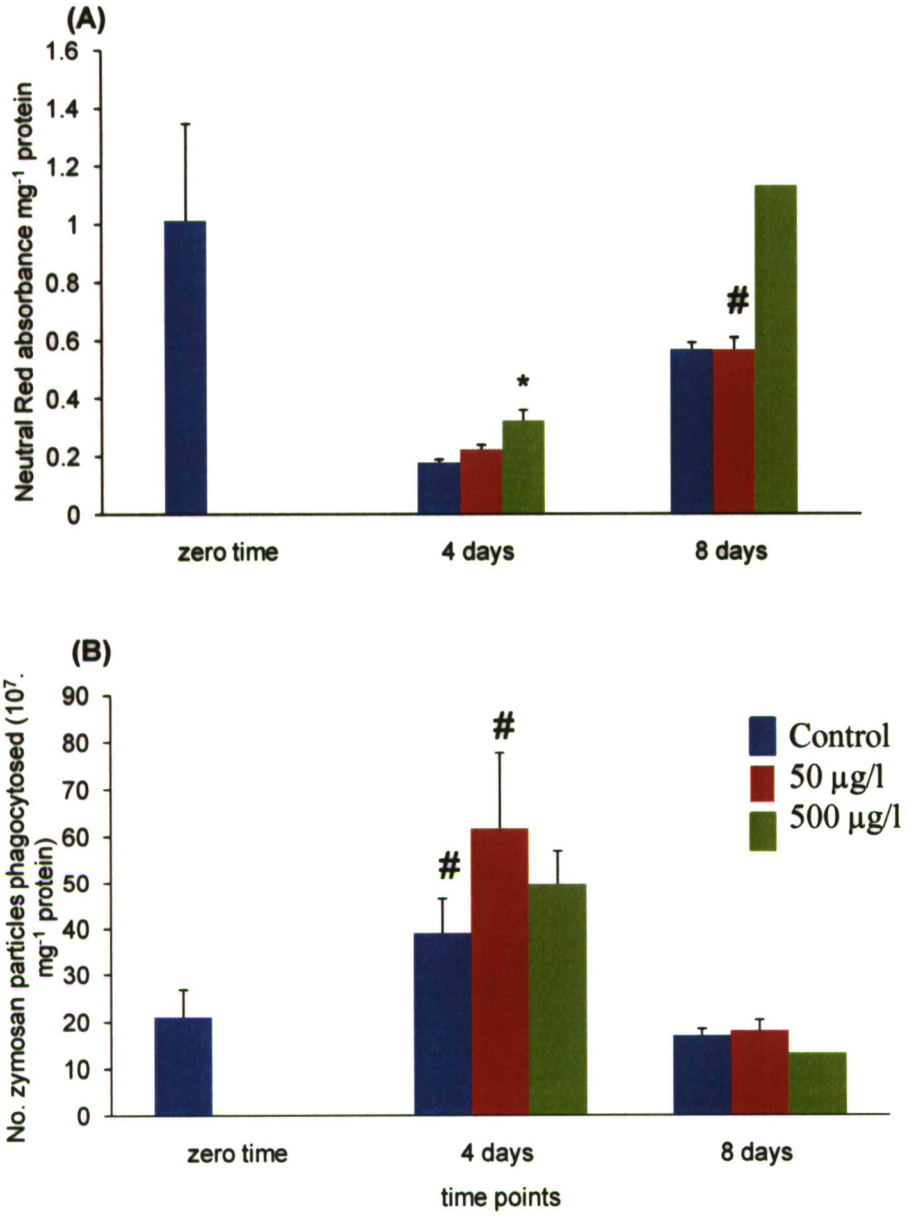

Fig. 3.1 Neutral Red uptake (a) and Phagocytic activity measured at 550 nm (b) in *Mytilus edulis* Control, 50 µg l^{-1} $HgCl_2$ mussels and 500 µg l^{-1} $HgCl_2$ mussels. Data are means ± S.E.M., $n = 6 - 9$ per treatment at each time point, except for 500 µg l^{-1} $HgCl_2$ mussels on day 8, $n = 1$. * indicates a significant difference from the respective control within each time point. # indicates a significant time-effect within treatment, $P \leq 0.05$, ANOVA or Kruskal Wallis.

3.3.2 Haemolymph chemistry during mercury exposure

Mussels were unfed during the experiment, and glucose levels were < 0.2 mmol l^{-1} in all animals, with most animals having glucose levels below the detection limit of the assay (< 0.01 mmol l^{-1}). Only one transient difference in glucose was found, with the mussels from the 50 μg l^{-1} Hg treatment showing higher glucose levels than 500 μg l^{-1} treatment or the controls at day 8 (One-way ANOVA, $P = 0.03$, $n = 5$). Haemolymph Na and K concentrations were unaffected by Hg exposure, with no time or treatment-dependent effects (ANOVAs, $P > 0.05$). Values ranged between 293 - 361 and 7.5 - 9.3 mmol l^{-1} for Na and K, respectively.

3.3.3 Histological alterations during Hg exposure

Gills from control animals showed normal architecture with typical fillibranch morphology showing the frontal and lateral cilia on the gill filaments. Gill pathology was absent in control animals with no evidence of oedema or erosion of the gill filaments (Fig. 3.2a). Hg exposure caused gill injury (Fig. 3.2c). The gills from 4 out of 6 animals examined showed evidence of hyperplasia and increased staining in the gill filaments. However, the haemolymph vessel in each filament was reduced or compressed suggesting the loss of haemolymph space from the filament with inflammation (Fig. 3.2c). The other 2 animals examined from the Hg treatment showed normal histology.

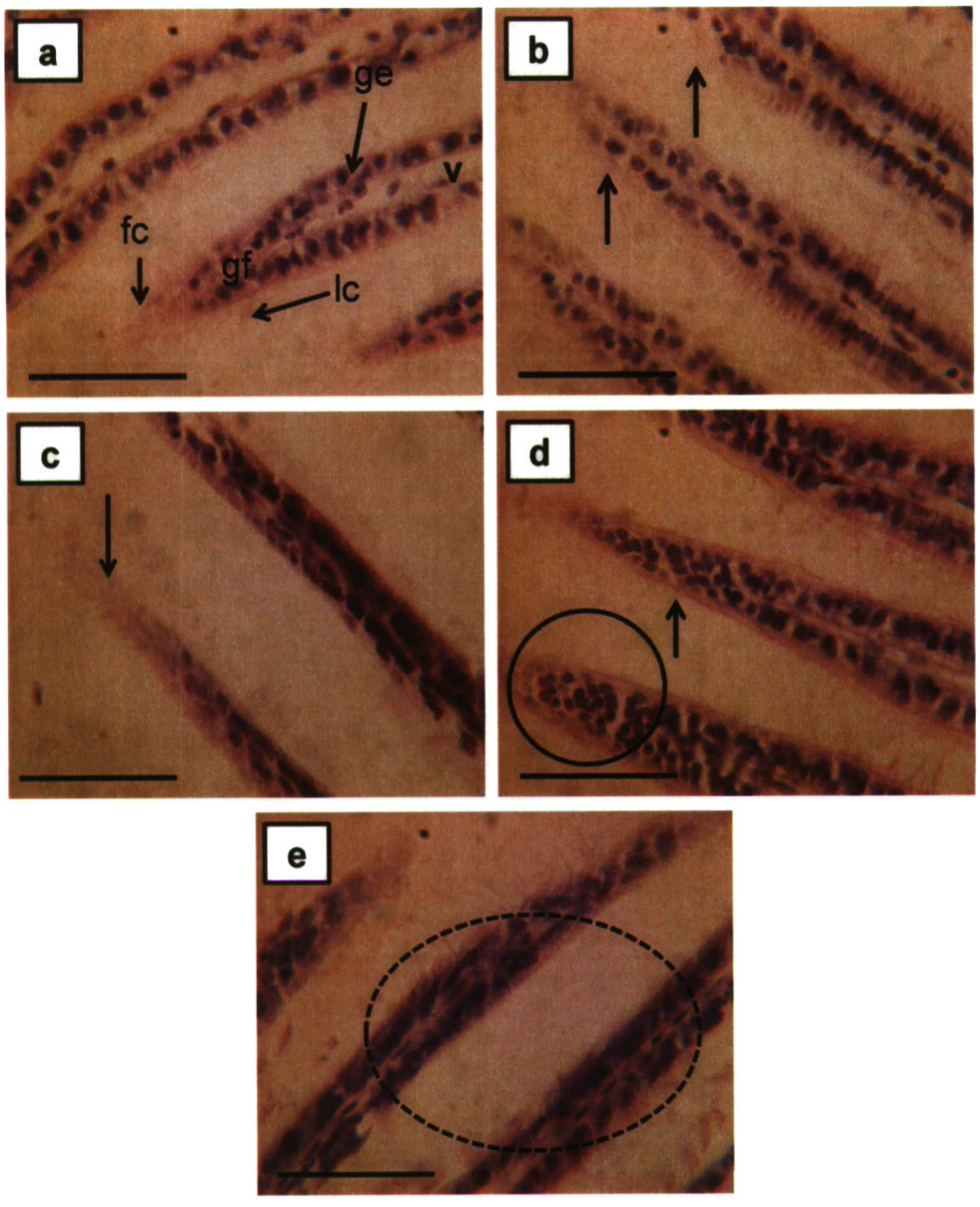

Fig. 3.2 Light micrographs of sections through gills of *M. edulis* showing histological structure of (a) control, (b) sham control, showing slightly less cilia in the gill filaments (arrows), (c) mussels treated with 50 µg l^{-1} Hg as $HgCl_2$, showing hyperplasia in cilia (arrows) and reduced haemolymph vessel (circle), (d) mussels injected with LPS, with less cilia (arrows) and hyperplasia (circle) (e) mussels injected with LPS and treated with 50 µg l^{-1} Hg, revealed reductions in cilia thickness, necrosis, and hypoplasia of the epithelium (dashed circle). Slides were stained with haematoxylin and eosin at 5-8 µm thickness. gf, gill filaments; fc, frontal cilia; lc, lateral cilia; ge, gill epithelium and v, haemolymph vessel. Scale bar, 50 µm.

The digestive gland of control animals showed normal architecture, with well defined digestive tubules and surrounding connective tissue (Fig. 3.3a). The epithelium in the intestine and the stomach appeared normal, with the clear ridges and grooves of the ciliated epithelium showing in the stomach (Fig. 3.3a). After the treatment, the connective tissue became necrotic. The nuclei of epithelial cells from the stomach and digestive tubules were unclear in some cells (Fig. 3.3c). Animals exposed to the 50 $\mu g\ l^{-1}$ Hg treatment showed pathology in the digestive gland at the end of the experiment (Fig. 3.3c and d). Also 5 animals out of 6 showed digestive tubules with necrosis, cells with diffuse nuclei, and epithelial cells with vacuoles. Some cells were so eroded that the distinction between cells in the intestinal epithelium was unclear. The epithelium of the stomach was also obstructed by haemocyte infiltrations, and this was observed in half of the sections examined.

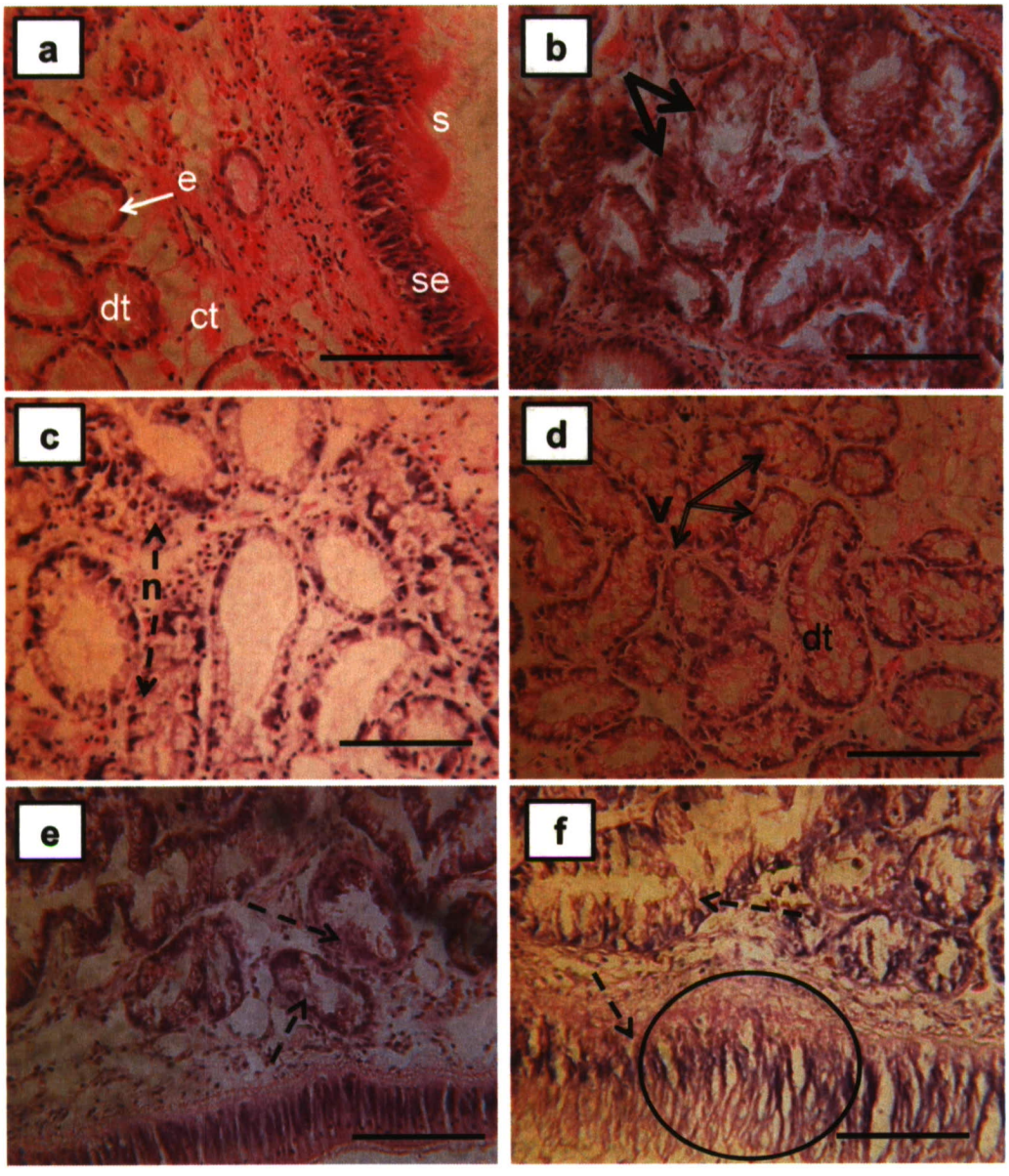

Fig. 3.3 Light micrographs of sections through digestive gland of *M. edulis* showing histological structure of (a) control, (b) sham control, with less well defined cell margins (thick arrows), (c) and (d) mussels treated with 50 μg l^{-1} Hg as $HgCl_2$, showing digestive tubules necrosis (dashed arrows) and vacuoles (double line arrows), (e) injected mussels with LPS, with tubules necrosis (dashed arrows) and (f) mussels injected with LPS and treated with 50 μg l^{-1} Hg, stomach epithelial necrosis (circle). Slides stained with haematoxylin and eosin at 5-8 μm thickness. Ct, connective tissue; dt, digestive tubules; s, stomach; se, stomach epithelium; e, epithelium. Scale bar, 100 μm.

The adductor muscle of control animals showed normal histology with the posterior adductor muscle consisting of muscle blocks, each made of distinct bundles of muscle fibres. Connective tissue was well defined around each bundle of muscle fibres. Myocytes appeared normal. There was no evidence of oedema, necrosis or other injuries in the controls (Fig. 3.4a). The adductor muscle of the mussels treated with 50 $\mu g\ l^{-1}$ Hg, showed lose of fibrous structure organization, necrosis, hydropic change (swelling), decrease in extracellular spaces and necrosis of connective tissue. Those abnormalities were found in at least 4 out of the 6 animals examined for each pathology (Fig. 3.4c).

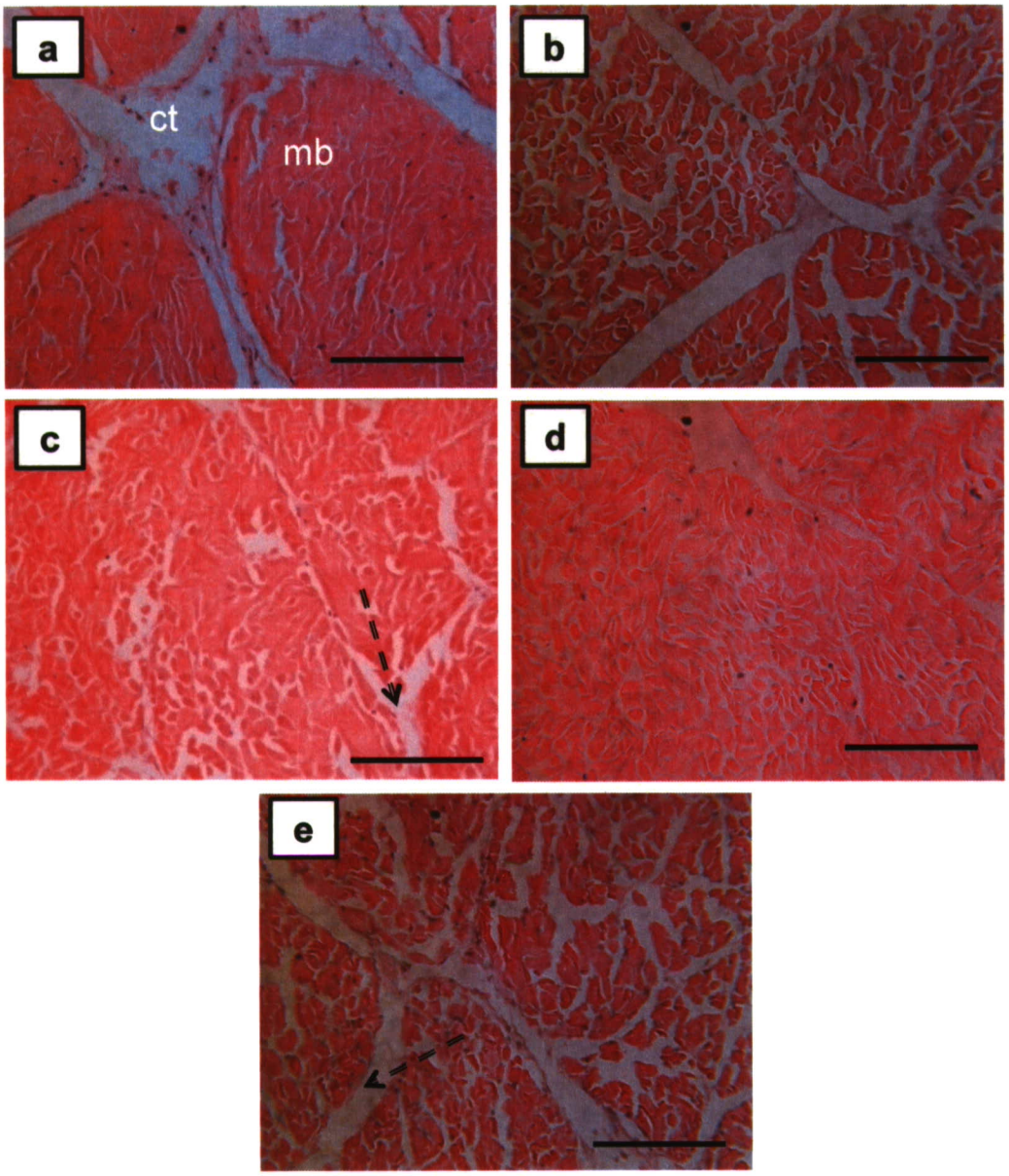

Fig. 3.4 Light micrographs of sections through adductor muscle of *M. edulis* showing histological structure of (a) control, (b) sham control, (c) mussels treated with 50 μg l^{-1} Hg as $HgCl_2$, showing loss of fibrous structure organization, decreases in extracellular space, and necrosis of connective tissue (dashed arrow), (d) mussels injected with LPS, with loss of muscular integrity and, (e) mussels injected with LPS and treated with 50 μg l^{-1} Hg, with hydropic change and connective tissue disruption (double line dashed arrow). Slides stained with haematoxylin and eosin at 5-8 μm thickness. mb, muscle bundle; and ct, connective tissue. Scale bar, 100 μm.

3.3.4 Hg accumulation, tissue electrolytes and immunological responses to Hg exposure after LPS treatment

In the second series of experiments, mussels were pre-injected with LPS, with and without subsequent exposure to 50 $\mu g\ l^{-1}$ Hg. There were no mortalities in the experiment. Hg concentrations in the sham-injected control animals and LPS injected animals (no Hg exposure) remained at or below the tissue detection limit (Table 3.3). Hg exposure after an injection of LPS caused large (2 orders of magnitude or more) increases in Hg content of tissues compared to either the sham-injected controls or LPS-injected animals without Hg exposure (Table 3.3). The Hg + LPS effect was statistically significant at all time points, compared to sham or LPS-injected controls (Kruskal Wallis, $P < 0.01$, time effect data not shown).

Similarly, the haemolymph showed a statistically significant Hg + LPS effects (Kruskal Wallis, $P = 0.01$; Table 3.3). Hg accumulation in the gills and adductor muscle in the Hg + LPS treatment was also significantly higher than that in the mercury alone trials ($P < 0.03$ or less, Kruskal Wallis/ANOVA).

Table 3.3 Mercury concentrations in gills, digestive gland, adductor muscle and haemolymph of *Mytilus edulis* exposed to 50 μg l^{-1} Hg as $HgCl_2$ after a pre-injection with LPS

Tissue	Hg Treatments (μg l^{-1})	Time (days) 1	4	8
Gills	Control	< 4.0	< 4.0	< 4.0
	LPS	< 4.0	< 4.0	< 4.0
	LPS + Hg	132.9 ± 17.8*	219.7 ± 12.6*#	229.9 ± 17.7*#
Digestive gland	Control	< 4.0	< 4.0	< 4.0
	LPS	< 4.0	< 4.0	< 4.0
	LPS + Hg	19.0 ± 4.9 *	93.0 ± 37.9 *#	43.7 ± 2.0*
Adductor muscle	Control	< 4.0	< 4.0	< 4.0
	LPS	< 4.0	< 4.0	< 4.0
	LPS + Hg	< 4.0	8.0 ± 1.3 *#	< 4.0
Haemolymph	Control	< 8.0	< 8.0	< 8.0
	LPS	< 8.0	< 8.0	< 8.0
	LPS + Hg	11.9 ± 5.3	24.5 ± 7.9 *	15.8 ± 4.2 *

Control; sham injected saline control (no mercury exposure). LPS; injected with LPS without Hg exposure. LPS + Hg; injected with LPS followed by Hg exposure. Data are means ± S.E.M. μg g^{-1} dw for tissues and mg l^{-1} for haemolymph, n = 6, mussels per treatment at each time point. * indicates a significant difference from the respective control within each time point. # indicates a significant difference in time within treatment, $P \leq 0.05$, ANOVA or Kruskal Wallis. Note, values for initial animals (time zero) at the start of the experiment were under detection limit for tissue Hg, 4 μg g^{-1} dw and 8 mg l^{-1} for haemolymph.

Na decreased in adductor muscle significantly because of the treatment with Hg when compared to the control mussels (Kruskal Wallis, P = 0.02), and Ca in digestive gland increased in LPS + 50 μg l^{-1} Hg over LPS alone and control mussels (ANOVA, P = 0.03). However, no significant differences were detected in tissue K content between the control and treated mussels within the investigated organs (ANOVA, $P > 0.05$, Table 3.4).

Table 3.4 Na, K and Ca concentrations in the gills, digestive gland and adductor muscle of *M. edulis* after 8 days exposure to 50 μg l^{-1} Hg as $HgCl_2$ after a pre-injection with LPS

Element (μg g^{-1} dw)	Treatments	Gills	Digestive gland	Adductor muscle
Na	control	21.4 ± 1.7	13.7 ± 1.5	17.5 ± 1.8
	LPS	29.3 ± 6.8	16.6 ± 2.6	15.3 ± 1.4
	LPS + Hg	24.5 ± 0.0	18.1 ± 4.4	11.4 ± 0.3*
K	control	9.9 ± 0.9	9.4 ± 0.9	6.9 ± 0.1
	LPS	11.1 ± 1.9	11.1 ± 1.9	6.3 ± 0.3
	LPS + Hg	15.2 ± 0.0	15.2 ± 0.0	6.1 ± 0.3
Ca	control	9.1 ± 2.1	5.3 ± 0.8	7.0 ± 0.8
	LPS	7.6 ± 1.4	6.6 ± 0.9	7.0 ± 1.0
	LPS + Hg	7.7 ± 0.0	10.8 ± 2.2*	4.4 ± 0.6

Data are means ± S.E.M. μg g^{-1} dry weight, n = 6 mussels per treatment at each time point. Sham control; animals injected with physiological saline instead of LPS (no Hg exposure). LPS; injected with LPS without Hg exposure. LPS + Hg; injected with LPS followed by Hg exposure. * indicates a significant difference from the control within each time point (ANOVA or Kruskal Wallis, $P < 0.05$).

Neutral red uptake by the haemocytes was unaffected by either LPS injection or the LPS + Hg treatment, except for a statistically significant rise in neutral red uptake by the LPS-injected group compared to either the sham-injected control or the LPS + Hg treatment on day 8 only (ANOVA, $P = 0.02$). Values for neutral red uptake on day 8 were (mean ± S.E.M., n = 6): 4.9 ± 0.4, 7.2 ± 0.7 and 5.7 ± 0.4 absorbance units mg^{-1} protein, for the control, LPS and LPS + Hg treatments respectively. A transient time-dependent increase in neutral red uptake was observed at day 4 in the control and LPS treatments (ANOVA, $P = 0.001$ and 0.04, respectively), but no Hg treatment-effect was observed (Fig. 3.5a).

There were no treatment-dependent effects on phagocytosis (ANOVA, $P = 0.7$ or greater) (Fig. 3.5b), or the ability of haemocytes to lyse sheep erythrocytes in the cytotoxicity assay (Kruskal Wallis, $P = 0.2$).

In the cytotoxicity assay, values at day 8 were (mean ± S.E.M., n = 6): 112 ± 1.2, 111 ± 1.1 and 113.8 ± 0.2 %, for the control, LPS and LPS + Hg treatments respectively. However LPS treatment did cause an increase in the number of circulating haemocytes compared to sham injected controls (Kruskal Wallis, P = 0.02), as expected when experimentally stimulating the immune response. This was not observed in the LPS + Hg group, indicating that Hg inhibited this response. Cell counts from animals at day 8 were (mean ± S.E.M., n = 6, cells ml^{-1} x 10^6); control, 2.07 ± 0.08; LPS treated, 3.18 ± 0.36, and LPS + Hg, 2.06 ± 0.2.

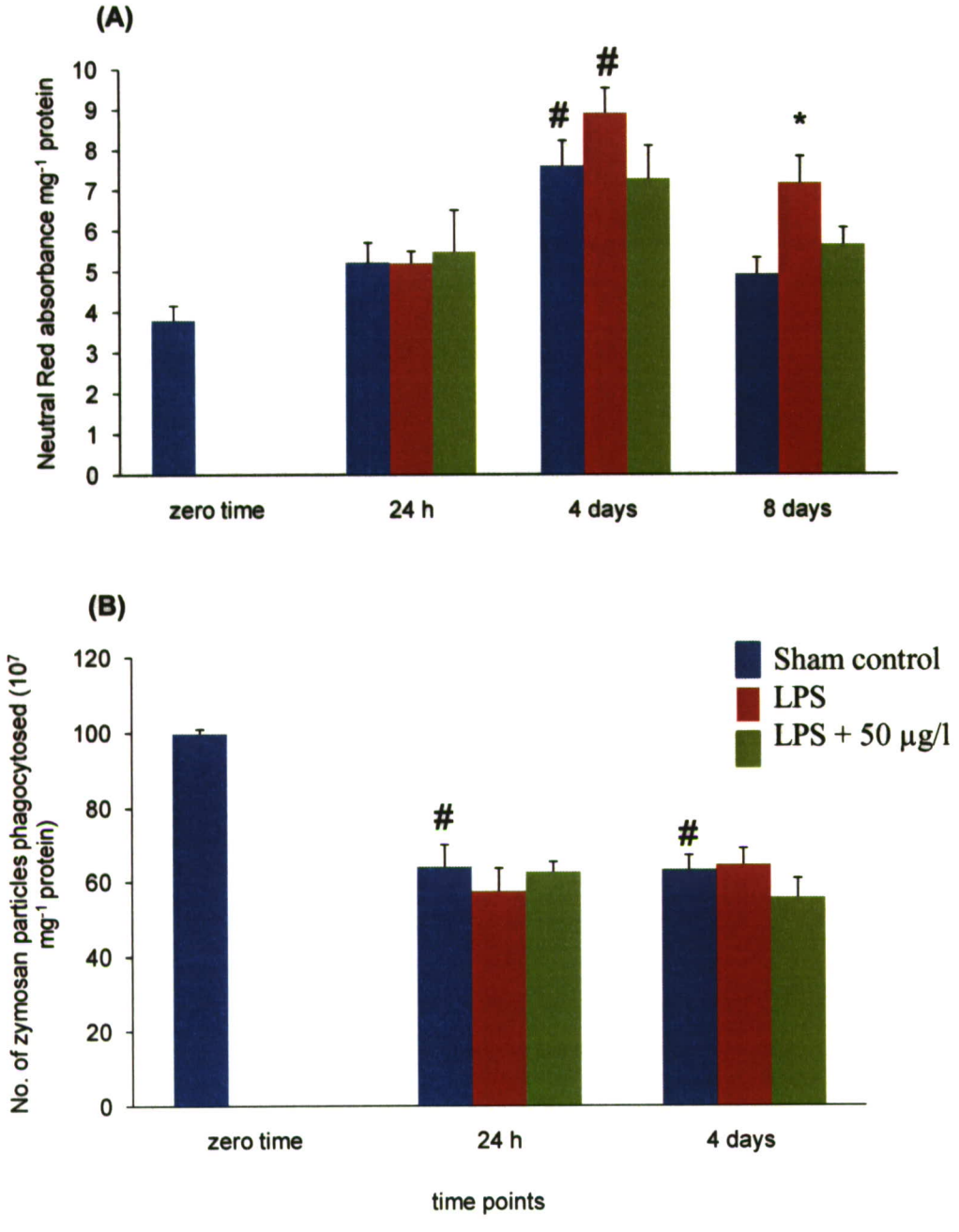

Fig. 3.5 Neutral Red uptake measured at 550 nm (a) and Phagocytic activity measured at 550 nm (b) in *Mytilus edulis*. Control, LPS injected mussels and LPS injected treated with 50 µg l^{-1} $HgCl_2$ mussels. Data are means ± S.E.M., $n = 6 - 9$ per treatment at each time point. * indicates a significant difference from the control within each time point. # indicates a significant time effect within treatment, $P \leq 0.05$, ANOVA or Kruskal Wallis.

3.3.5 Haemolymph chemistry during mercury exposure after LPS

Haemolymph electrolyte concentration showed no treatment effects (Table 5.3). There was a statistically significant rise in glucose in the LPS-injected group compared to controls on day 1 only, and a time-effect within the LPS + Hg group only by day 8 (Table 3.5). However, such micromolar changes in glucose are unlikely to be of biological importance.

Table 3.5 Glucose, Na and K concentrations in haemolymph of *M. edulis* after an injection of LPS, with or without subsequent expose to 50 $\mu g\ l^{-1}$ Hg as $HgCl_2$

Parameter ($mmol\ l^{-1}$)	Treatment	Time (days)		
		1	4	8
Glucose	sham control	0.8 ± 0.02	0.8 ± 0.06	0.9 ± 0.06
	LPS	1.2 ± 0.2*	0.7 ± 0.02	0.8 ± 0.04
	LPS + Hg	0.7 ± 0.04	0.08 ± 0.04	0.97 ± 0.09#
Na	sham control	503.3 ± 6.1	500.0 ± 5.8	485.0 ± 6.5
	LPS	488.0 ± 10.7	498.0 ± 9.5	440.0 ± 48.1
	LPS + Hg	502.0 ± 13.8	493.0 ± 4.8	476.7 ± 8.8
K	sham control	11.4 ± 0.2#	11.2 ± 0.3#	11.4 ± 0.2#
	LPS	12.0 ± 0.2	11.5 ± 0.3	11.7 ± 1.2
	LPS + Hg	11.6 ± 0.3	12.3 ± 0.4	11.7 ± 0.5

Data are means ± S.E.M., n = 6 per treatment at each time point. Sham control; an animal injected with physiological saline instead of LPS (no Hg exposure). LPS; injected with LPS without Hg exposure. LPS + Hg; injected with LPS followed by Hg exposure. * indicates significant difference from the respective control. # indicates a significant difference in time within treatment ($P < 0.05$, Kruskal Wallis or ANOVA). Note, values for initial animals (time zero) at the start of the experiment were 0.9 ± 0.03, 477.5 ± 9.6, 10.7 ± 0.1 for glucose, Na, and K, respectively.

Superoxide dismutase was measured in the extracellular fluid component of the haemolymph and in the haemocytes to estimate intracellular SOD activity. There was a statistically significant decrease of intracellular SOD at 24 h with both LPS and LPS + Hg treatments having lower enzyme activities than the sham injected controls; and with the LPS + Hg treatment also being lower than the LPS injected group (Kruskal Wallis, $P = 0.006$). Intracellular SOD at 24 h was (mean ± S.E.M., $n = 6$); 167.0 ± 38.3, 47.8 ± 6.6, 25.5 ± 3.6 units ml^{-1} haemolymph in controls, LPS, and LPS + Hg groups respectively. This effect was lost by the end of the experiment. There were no changes in extracellular SOD activity with values at the end of the experiment (mean ± S.E.M., $n = 6$); 721.4 ± 289.5, 470.2 ± 28.5, 894.5 ± 248.6 units ml^{-1} haemolymph in controls, LPS, and LPS + Hg groups respectively.

3.3.6 Histological alterations during Hg exposure after LPS treatment

Gills from saline injected controls showed normal histology. There were some subtle effects of LPS treatment on the gills, with some filaments showing slightly less cilia (Fig. 3.2d). Mussels from the LPS + Hg treatment showed a reduction in cilia thickness, necrosis, and hypoplasia of the gill epithelium.

Some mild histological abnormalities were found in the digestive gland of saline-injected controls. These abnormalities included foci of cells with slightly less well defined cell margins on some digestive tubules (Fig. 3.3b). This pathology was not wide-spread and all other structures were normal. All mussels injected with LPS showed alterations in the digestive gland, especially the digestive tubules. The digestive tubules showed necrosis, diffuse nuclei, and some foci where there was poor distinction of epithelial cells (Fig. 3.3e). Stomach from 2 mussels was also obstructed with an inflammatory infiltrate of haemocytes. Digestive gland from the LPS + Hg treatment showed similar, but generally more severe pathologies compared to the LPS-injected

group (Fig. 3.3f). Five out of 6 animals in the LPS + Hg group showed both digestive tubules and stomach epithelia with necrosis, and no clear distinction of epithelial cells. In the stomach, 5 out of 6 animals showed epithelial necrosis with fibrosis and inflammation in the connective tissue (Fig. 3.3f).

Saline injection had no effect on the posterior adductor muscle (Fig. 3.4b). However, all the muscles examined from the LPS injected animals showed some loss of muscle fibre integrity (Fig. 3.4d). In the LPS + Hg treatment the pathology was more severe, and in 80 % of the mussels examined a decrease in extracellular spaces, disruption of connective tissue, and hydropic change (swelling) in the muscle bundles was observed (Fig. 3.4e).

3.4 Discussion

There have been some studies on bivalves that report potential immunotoxic effects of Hg on haemocytes, especially *in vitro* (e.g., Cheng and Sullivan, 1984; Bolognesi et al., 1999; Sauvé et al., 2002; Duchemin et al., 2008). However this study is one of the first *in vivo* experiments on *M. edulis* to measure Hg accumulation, immunological properties of the haemocytes, histopathology, and effects of LPS within the same investigation. We show a number of transient changes in immunological functions of haemocytes during Hg exposure, organ pathologies, and find that pre-treatment with LPS exacerbates Hg toxicity.

3.4.1 Effect of Hg exposure alone

Hg exposure was confirmed by measurements of total Hg in the tissues (Table 3.1). The 50 $\mu g\ l^{-1}$ Hg concentration was expected to be firmly in the sub-lethal range, and the 500 $\mu g\ l^{-1}$ concentration to be approaching the toxicity threshold. This seemed to be the

case with only one mortality at the lower Hg concentration, but a cumulative mortality (notably only after day 6) at the higher concentration. Thus the focus of the immunological measurements and other investigations were therefore mainly on the lower Hg concentrations, and also for the subsequent LPS experiments. The mussels exposed to 500 $\mu g\ l^{-1}$ Hg did accumulate Hg, but also spent much of the time with their shells closed. The gills of these animals showed severe pathology, and tissue hypoxia may therefore be a factor in the mortalities from the 500 $\mu g\ l^{-1}$ Hg treatment.

Background levels of Hg in the tissues were a few $\mu g\ g^{-1}$ dw (Table 3.1) and these are consistent with previous reports on *Mytilus* species (Canesi et al., 1999). The gills showed the highest levels of Hg accumulation as expected for an aqueous exposure (Table 3.1). This general pattern of Hg accumulation is also consistent with previous studies on Hg exposure in bivalves (e.g., Canesi et al., 1999).

Hg levels also increased in the haemolymph (Table 3.1), but there were generally no effects on haemolymph electrolyte levels. Haemolymph glucose levels also remained low in this experiment, as expected for animals that have not been fed for several days. Thus any changes in the responsiveness of haemocytes would not be due to overt osmotic stress or associated deterioration in haemocyte integrity, and this was supported by the neutral red measurements. Neutral red retention is regarded as an indicator of lysozomal integrity, and therefore cell health (e.g., Lowe et al., 1995), and this showed no effect of Hg exposure. Thus the haemocytes could be regarded as reasonably healthy. Neutral red content of haemocytes increased at day 4 of exposure in the 500 $\mu g\ l^{-1}$ treatment (Fig. 3.1a), but apart from that one time point, there were no treatment-effects of Hg. The mean neutral red uptake was higher and more variable in the animals at time zero (stock animals, well fed), and decreased over time in the controls (animal not fed during the experiment), suggesting that the response of the assay may also be influenced by nutritional status. This does raise an important point

about toxicity testing protocols involving immunotoxic end points, and this experiment is much longer than previous studies on *Mytilus edulis* (usually 24 h Smith et al., 2000). The cells showed normal phagocytosis ability in the controls, there was no clear Hg-dependent effect on the ability of haemocytes to phagocytose zymosan particles (Fig. 3.1b). Gagnaire et al. (2004) also report no effect of $HgCl_2$ on phagocytic activity on haemocytes of the Pacific oyster (*Crassostrea gigas*) exposed to 2-200 $\mu g\ l^{-1}$ Hg for 24 h *in vitro*. In our study the ability of haemocytes to lyse sheep red blood cells (the cytotoxicity assay) showed no clear Hg-dependent effects. The background level of cytotoxicity ability (88 %) in our present study is similar to previous reports (e.g., 79 %, Rickwood and Galloway, 2004) suggesting the cells were functional; but just not responding to Hg. Haemocyte counts were also unaffected. Taken together, these observations suggest that Hg neither inhibits nor stimulates the haemocytes compared to unexposed controls.

However, some severe pathologies consistent with inflammation occurred (Figs. 3.2, 3.3 and 3.4), suggesting that the haemocytes were not protecting the organism. All the organs (adductor muscle, digestive gland and gills) showed histological change associated with Hg exposure. The 500 $\mu g\ l^{-1}$ Hg concentration caused severe pathology to the gills (complete disruption of the epithelium, data not shown), and probably caused the death of the animals (consistent with previous reports, Gregory et al., 1999). There are no reports of digestive gland histology during Hg exposure in bivalves, and in our experiment necrosis and severe disruption of the epithelia was observed suggesting that 50 and 500 $\mu g\ l^{-1}$ Hg exposure would compromise digestive functions. The gills showed loss of the lateral and frontal cilia (Fig. 3.2), and this would presumably compromise the capture, transport, and sorting of food particles. The gills are also an osmoregulatory organ, but this level of injury did not appear to compromise the haematology. The cellular injuries to the gill epithelium included some necrosis of the

cilia, and hyperplasia of the gill tissue. Gregory et al. (1999) noted necrotic cells and an unusual increase (assumed hyperplasic change) in the number of cilia on the lateral surfaces of gills filaments in *Perna perna* exposed to $HgCl_2$, but these effects have not been reported previously for *M. edulis*. We also observed injury to the posterior adductor muscle during Hg exposure, characterised by loss of muscle fibre bundle integrity and some inflammation (Fig. 3.4). Clearly, the adductor muscles are responsible for opening and closing the shell, and so damage to this muscle may compromise feeding behaviour or ventilation, and also increase the risk of predation if the shell cannot be tightly closed.

3.4.2 Effect of LPS pre-treatment prior to Hg exposure

The LPS pre-treatment was a single LPS injection prior to Hg exposure. The effects of LPS injections are known to persist for at least 17 days in mussels (Hernorth, 2003b), and it is therefore clear that the animals in the LPS + Hg treatment in the current experiment (8 days) would have been subjected to biological activity of both substances at the same time. Injection of LPS alone compared to the sham saline injection had no effects of the neutral red uptake (Fig. 3.5a), or haemolymph Na and K levels (Table 3.4). However, it did raise haemolymph glucose levels, perhaps indicating the initiation of a stress response (Lorenzon et al., 1997). In addition, there was histological change in the gills (hypoplasia, Fig. 3.2), some occasional foci of necrosis in the digestive gland (Fig. 3.3), with evidence of haemocyte infiltration indicating an immune response. In this experiment the purpose of the LPS injection was to challenge the organism and stimulate an immune response (Beutler, 2005). This appears to be the case with haemocyte infiltration into the tissues, but without loss of integrity of the haemocytes themselves, or the haemolymph. There were also some changes in the adductor muscle consistent with mild inflammation (Fig. 3.4), and this to be expected at the site of LPS

injection. However, the areas of erosion in the digestive gland after LPS injection were less expected. The gut epithelium in mammals is well known for being sensitive to immunological status and is affected by LPS (e.g., Ismail and Hooper, 2005). The histological observations (Fig. 3.3) suggest this is also the case for mussels.

There are several possible explanations for the interaction of LPS with Hg exposure including; (i) the LPS injection should stimulate the immune response and prime the organism so that it can be more easily defended from the inflammatory effects of Hg, or (ii) the LPS injection is stressful and challenges the immune system so that the addition stress of Hg exposure increases tissue injury compared to the effects of either LPS or Hg alone. A beneficial priming effect seems unlikely because the pathology in the LPS + Hg treatment was generally worse than LPS alone (Figs. 3.2, 3.3 and 3.4). The weight of evidence in this study suggests that LPS probably exacerbates the effects of Hg in terms of tissue injury and inflammation, but has only minor effects on the haemolymph and haemocytes. The absence of any differences between LPS injection alone and LPS + Hg on neutral red uptake, as well as haemolymph Na, K and glucose suggests no additional effects of Hg compared to LPS alone on the haemolymph. However, the histology (Figs. 3.2, 3.3 and 3.4) suggests tissue injuries that are greater in the LPS + Hg treatment compared to either Hg or LPS alone. Thus the tissues are being injured and the haemocytes are not defending the organism from this tissue inflammation and erosion. One possible explanation for the lack of a haemocyte response to Hg is that the cells have insufficient levels of the superoxide anion which are integral to phagocytosis and other cell killing processes. Superoxide anions can be removed from cells by the activity of SOD which converts the superoxide anions to hydrogen peroxide. However, in this study SOD activity did not increase in either the LPS or LPS + Hg treatments, and at one time point actually decreased for a while.

Alternatively, the haemocytes may simply not have the metabolic energy to perform these functions during Hg exposure, even in the presence of LPS stimulation.

3.5 Conclusion

M. edulis accumulates Hg in the gills, digestive gland, adductor muscle, and haemolymph. This Hg accumulation is associated with inflammation and organ pathology, but without loss of integrity of haemocytes. The physiological integrity of the haemolymph is preserved in terms of electrolytes and cell counts, but despite widespread organ pathologies, the haemocytes do not show immune stimulation or suppression during Hg exposure at the concentrations and exposure times used here. The tissue inflammation is exacerbated with an LPS pre-treatment prior to Hg exposure, suggesting that pre-sensitising the organism makes the toxic response to Hg worse, but it does not "switch on" or enhance the immune functions of haemocytes. It remains unclear whether the haemocytes are unable to respond, or simply not responding to Hg, and further work on the immunological signalling pathways in haemocytes during Hg exposure is needed to resolve this, along with the measurement of inflammatory markers (e.g., cytokines or their analogues) in the haemolymph.

Chapter 4

Tissue injury and cellular immune responses to cadmium chloride exposure in the common mussel *Mytilus edulis*: modulation by lipopolysaccharide

Chapter 4

Tissue injury and cellular immune responses to cadmium chloride exposure in the common mussel *Mytilus edulis*: modulation by lipopolysaccharide

Abstract

The immunotoxic effects of cadmium (Cd) exposure in bivalves are poorly understood and whether or not stimulation of the immune system exacerbates Cd toxicity is unclear. The mussel, *Mytilus edulis*, was exposed to 20 or 50 μg l^{-1} total Cd for up to 11 days compared to no added Cd controls to assess immune and other physiological responses. Selected experiments were then repeated in the presence of a lipopolysaccharide (LPS) challenge with and without subsequent Cd exposure. Immune functions of haemocytes, haematology, haemolymph glucose and ion content, as well as superoxide dismutase (SOD) activity, and organ pathology were measured. Cd accumulated mainly in digestive gland and gills, and to a lesser extent in the adductor muscle. Exposure to 20 μg l^{-1} Cd alone caused a transient modulated of phagocytosis and increased neutral red uptake (Kruskal Wallis, P = 0.002). The higher Cd concentration also increased cytotoxicity, and decreased haemocyte count. Changes in haemolymph Na, K and glucose were small or neglible. Histopathological examination showed tissue injuries consistent with inflammation and necrosis in the gills, digestive gland and adductor muscle during Cd exposure alone. LPS injection alone, and LPS + Cd, caused an increase in the number of circulating haemocytes by the end of the experiment (Kruskal Wallis, P = 0.01), and a transient rise in phagocytosis at day 4 (ANOVA, P = 0.001). The LPS + Cd treatment also caused transient changes in neutral red uptake, and in the cytotoxicity of haemocytes compared to controls. Intracellular SOD activity did not change in haemocytes under any treatment. Tissue inflammation and pathology was greatly increased by the effect of Cd exposure with an LPS injection compared to either

treatment alone. I conclude that immunostimulation with LPS can greatly increase Cd-related organ pathologies, but does not necessarily alter the responses of haemocytes.

4.1 Introduction

The ecotoxicology and chemistry of cadmium (Cd) has been extensively studied in marine systems, with Cd being regarded as a non-essential and toxic metal (e.g., Webb 1979; Phillips 1980; Jensen and Bro-Rasmussen 1992). Cd is acutely toxic to marine bivalves, with LC_{50} estimates of around 1.6 mg l^{-1}, depending on the hydrated ionic radius of the metal (van Kolck et al., 2008). Bivalves from contaminated ecosystems can show appreciable Cd accumulation (e.g., *Mytilus edulis*, 7.1 μg g^{-1} dry weight, dw, Roesijadi et al., 1984). Sub-lethal effects include disturbances to respiration (Poulsen et al., 1982), alterations in haemolymph chemistry and osmoregulation (eg., Hemelraad et al., 1990), and specific effects on calcium homeostasis such as changes in intracellular Ca in haemocytes (Faubel et al., 2008). The haemocytes are an important component of the invertebrate immune system (review, Galloway and Handy, 2003). The immune system of bivalves relies on the circulating haemocytes being able to penetrate tissue in response to antigens, and the immune system has both innate and humoral components, in which haemocytes play a key role (Canesi et al., 2002). The haemocytes have phagocytic functions which are analogous to those in vertebrate animals (Wootton et al., 2003).

Immunotoxicity associated with trace metals exposure has been reported in bivalves (Pipe and Coles 1995; Pipe et al., 1999; Galloway and Depledge 2001). Immunotoxicity from Cd exposure has been relatively well documented in mammals (e.g., Sant'Ana et al., 2005). There are some reports on fish (e.g., Zelikoff et al., 1995), and a few reports on invertebrate species *in vivo* (Coles et al., 1995; McIntosh and Robinson 1999; Cherkasov et al., 2007). These *in vivo* reports show that both Cd

clearance and haemocyte turnover are incidental (around 25 days, McIntosh and Robinson, 1999) and that the Cd toxicity to haemocytes is a function of temperature (Cherkasov et al., 2007). However, *in vitro* reports on Cd toxicity to haemocytes show suppression and stimulation of immune functions in bivalves, depending on the experimental conditions (e.g., Anderson et al., 1992; Brousseau et al., 1999). Cd is also known to have direct effects on the cell biology of haemocytes, and can alter ATP levels as well as initiating apoptosis (e.g., in the Eastern oyster, *Crassostrea virginica*, Sokolova et al., 2004).

Lipopolysaccharides are found on the external membranes of Gram negative bacteria and are known to stimulate immune responses in eukaryote organisms by several methods, including the activation of signal transduction pathways in macrophages (Ulevitch and Tobias, 1995). LPS at high concentrations can cause over-stimulation of the immune system and acute hypersensitivity reactions, leading to mortality, for example, in fish exposed to LPS in algal blooms (Best et al., 2002). However at low μg concentrations, LPS can be used as a tool to give a controlled stimulation of the immune system (e.g., Coa et al., 2004).

The aim of the present study was to conduct an *in vivo* study on the immunotoxic effects of Cd with *M. edulis*. The effects of Cd exposure were investigated by performing two series of experiments within the study design. The first trials explored the concentration and time dependence of Cd-induced immunotoxicity by measuring the immune responses of *M. edulis* haemocytes for up to 11 days, rather than just a few hours or days as in previous studies. We also measured the composition of the haemolymph (electrolytes, cells, glucose levels) so that the general health of the body fluids, and the organism (organ histology), could be placed in context of the immune-related effects of Cd. In the second series, we explored the influence of a LPS injection prior to Cd exposure. The working hypothesise were that an LPS injection may prime

the immune system so that the organism is protected from the effects of Cd, or alternatively, that LPS pre-treatment makes the organism more sensitive and exacerbates the immunotoxic effects of Cd.

4.2 Materials and methods

4.2.1Experimental design

The experiment was split into two consecutive trials for logistic reasons, and this was absolutely essential so that all the haemocyte assays could be performed fresh, immediately on the day of sampling. The first trial was conducted to explore the toxic effects of exposure to 0 (no added Cd control), 20 or 50 total Cd $\mu g\ l^{-1}$ as $CdCl_2$ for up to 11 days in mussels *in vivo*. Measurements included phagocytosis, neutral red uptake , and cytotoxicity assays to assess the immune functions of haemocytes from the mussels, as well as haemolymph Na, K, glucose (see below), on days zero, 4, 8, and 11 of exposure. A total of 90 mussels were randomly allocated to the tanks (10 animals/tank, in triplicate, 30 animals/treatment). At least 6 animals at each time point (2/tank) were used for each assay, with an additional 6 animals/treatment also being collected for histology at the end of the exposure (day 11). All tanks were acid-washed (10 % HNO_3) and rinsed in clean seawater prior to use. Each tank (L: 30.3 cm, W: 10 cm and H: 20.5 cm) contained 10 litres of filtered sea water and was maintained at a constant temperature (14 ± 1 °C). Cd stock solutions were prepared by dissolving 0.065 $g\ l^{-1}$ and 0.163 $g\ l^{-1}$ of $CdCl_2$ in acidified Millipore water respectively, and 5 ml of one of these stock solutions was added to the appropriate tank to give nominal total Cd concentrations of 20 and 50 $\mu g\ l^{-1}$ respectively. This dose range was selected after considering previous reports of sublethal Cd toxicity to *M. edulis* (e.g., Strömgren 1982). A semi-static exposure method was used and tanks were re-dosed every 24 h with 5 ml of the appropriate stock solution, after a 100 % water change. The exposures

were confirmed by measured total Cd concentrations in the tanks (see below) on sampling days and were (mean ± S.E.M., $n = 27$): 15.5 ± 3.4 and 53.3 ± 4.2 μg l^{-1} for the 20 and 50 μg l^{-1} treatments respectively. Background Cd level in the control filtered sea water was < 1 μg l^{-1}. Seawater quality was: % dissolved oxygen (102 ± 0.2), pH (7.98 ± 0.01), total ammonia (0.01 ± 0.01 mg l^{-1}) and salinity (34.1 ± 0.01 ppt). Temperature, dissolved oxygen, ammonia and pH were measured every day and remained within the limits outlined above.

The second trial aimed to explore the effects of LPS (from *E. coli*, 055: B5) with and without Cd exposure. The 20 μg l^{-1} Cd concentration was selected for this trial. The measured endpoints and numbers of mussels/treatment used (in triplicate) were identical to the first series, except that the experiment was shorter with sampling at 0, 24 h, 4 and 8 days, with histology and SOD activity in the haemocytes also measured (see below) at the end of the exposure (day 8). Mussels were divided into 3 treatments; sham-injected saline control (no LPS or Cd), LPS injected in the posterior adductor muscle (LPS alone, no Cd), and LPS injected followed by Cd exposure (LPS + Cd exposure). The LPS stock (obtained from Sigma) contained 2 mg ml^{-1} LPS and each animal was injected with either 50 μl of saline (control) or the LPS to give a total LPS dose of 100 μg per animal. This dose was selected after reviewing doses used in previous experiments with *M. edulis* (Smith et al. 2000). Sham injections were done with 50 μl of phosphate buffered saline (PBS tablets, Sigma, P-4417).

Collection of mussels and stock aquaria, haemolymph chemistry, haemocyte counts, and tissue collection, metal analysis, immunological assays, superoxide dismutase (SOD) and histology assays were explained in details in materials and method, Chapter 2.

4.3 Statistical analysis

All data were analysed using Statgraphics 5.1 Plus software. Data are expressed as means of triplicates ± S.E.M. for n = 6 mussels per treatment, unless otherwise stated. See Chapter 2, materials and methods for details.

4.4 Results

4.4.1 Cd accumulation and tissue electrolytes during Cd exposure

The first experiment explored the effects of exposure to either 20 or 50 μg l^{-1} Cd. There were no mortalities in the controls or Cd treatments during the experiment. Cd exposure was confirmed by Cd accumulation in the tissues (Table 4.1). The adductor muscle, digestive gland and gills of animals exposed to Cd demonstrated highly significant increases in tissue levels of Cd compared to the control; with Cd concentrations being highest in the digestive gland > gill > posterior adductor muscle (Table 4.1). Two-way ANOVA found significant time and treatment effects (p values < 0.0001). Tissues showed the expected time-dependent increase in Cd concentration during exposure and the Cd-effect on accumulation was significant for most time points compared to the controls (Kruskal Wallis, P = 0.01 or less) for all tissues during the experiment.

Table 4.1 Total Cd concentrations in digestive gland, gills, and adductor muscle of *Mytilus edulis* in two separate experimental trials, Cd exposure alone, and Cd exposure after an LPS injection

Tissue	Cd Treatment ($\mu g\ l^{-1}$)	Time (days)			
		1	4	8	11
Cd alone trial					
Digestive gland	Control		< 0.46	1.82 ± 0.37	2.15 ± 0.17 *#
	20		4.59 ± 0.57 *	29.2 ± 2.0 *#	57.2 ± 5.56 #
	50		28.72 ± 3.9 *	85.8 ± 9.15 *#	101.1 ± 11.44
Gills	Control		< 0.46	0.71 ± 0.13 #	1.21 ± 0.19 #
	20		0.78 ± 0.11	6.82 ± 0.82	27.64 ± 9.43 *#
	50		28.2 ± 3.13 *	27.18 ± 3.96 *#	49.06 ± 5.29 *
Adductor muscle	Control		< 0.46	1.02 ± 0.12 #	1.75 ± 0.26 #
	20		< 0.46	1.22 ± 0.09	4.63 ± 1.22 #
	50		4.42 ± 0.75*	11.27 ± 0.48 *	19.86 ± 3.42*#
LPS + Cd trial					
Digestive gland	Sham control	< 0.46	1.36 ± 0.1	1.75 ± 0.25 #	
	LPS	< 0.46	0.75 ± 0.08	1.42 ± 0.08 #	
	LPS + Cd	3.14 ± 0.23	16.5 ± 2.86 *	27.39 ± 4.82 *	
Gills	Sham control	< 0.46	0.7 ± 0.12 #	1.89 ± 0.19 #	
	LPS	< 0.46	< 0.46	< 0.46	
	LPS + Cd	2.6 ± 0.25 *	7.39 ± 1.24 *	16.59 ± 1.34 *#	
Adductor muscle	Sham control	< 0.46	0.59 ± 0.06 #	1.02 ± 0.1 #	
	LPS	< 0.46	0.7 ± 0.12	< 0.46	
	LPS + Cd	1.13 ± 0.13	1.09 ± 0.13	3.06 ± 0.42 #	

Data are means ± S.E.M. μg g^{-1} dry weight, n = 6 per treatment at each time point. In the Cd alone trial animals were exposed to 20 or 50 μg l^{-1} Cd as $CdCl_2$ compared to a no-added Cd control. In the LPS + Cd trial, animals were given a LPS injection prior to exposure to 20 μg l^{-1} Cd. * indicates a significant difference from the control within each time point (ANOVA or Kruskal Wallis, $P < 0.05$). # indicates a significant difference from the previous time point within treatment ($P < 0.05$, ANOVA or Kruskal Wallis). Note, < 0.46 indicates where values were at or below the Cd detection limit of 0.46 μg g^{-1} dry weight for tissues. Values for initial stock animals (time zero) at the start of the experiment were 1.49 ± 0.16, 0.65 ± 0.08, < 0.46 μg g^{-1} dw for digestive gland, gills, and adductor muscle respectively in the Cd trial. Values for the initial stock animals in the LPS + Cd trial were 0.58 ± 0.13, < 0.46, and < 0.46 μg g^{-1} dw respectively.

Tissue electrolytes like, Na, K and Ca were measured in digestive gland, gills and adductor muscle on day 11. No significant differences in K concentration detected between the control and treated mussels with 20 or 50 μg l^{-1} Cd in all tested organs (ANOVA, $P > 0.05$). However, gills and adductor muscle recorded a significant decrease of Na levels in the 20 μg l^{-1} Cd treated animals compared to the control (ANOVA/Kruskal Wallis, $P = 0.01$). Both treatments with Cd caused reductions in Ca concentration in the digestive gland and adductor muscle, but in gills, the treatment with 20 μg l^{-1} Cd caused the reduction of Ca levels compared to the control group (ANOVA/Kruskal Wallis, $P = 0.02$) (Table 4.2).

Table 4.2 Total Na, K and Ca concentrations in digestive gland, gills, and adductor muscle of *Mytilus edulis* after 11 days of exposure to 20 and 50 μg l^{-1} Cd as $CdCl_2$

Element (μg g^{-1} dw)	Treatments	Digestive gland	Gills	Adductor muscle
Na	Control	22.9 ± 2.3	53.4 ± 14.3	26.8 ± 3.1
	20 μg l^{-1}	17.7 ± 3.5	18.8 ± 2.8 *	11.9 ± 2.8 *
	50 μg l^{-1}	18.6 ± 1.0	46.5 ± 4.1	19.3 ± 2.1
K	Control	11.3 ± 1.1	12.5 ± 4.1	5.5 ± 0.2
	20 μg l^{-1}	9.6 ± 1.6	7.1 ± 1.1	4.4 ± 0.9
	50 μg l^{-1}	9.2 ± 0.3	10.6 ± 1.0	6.3 ± 0.3
Ca	Control	13.7 ± 1.6	18.9 ± 4.8	22.6 ± 3.2
	20 μg l^{-1}	7.7 ± 0.5 *	7.4 ± 0.9 *	9.4 ± 3.2 *
	50 μg l^{-1}	9.2 ± 0.3 *	14.2 ± 1.4	12.7 ± 1.7 *

Data are means ± S.E.M. μg g^{-1} dry weight, n = 6 mussels per treatment. * indicates a significant difference from the control within each time point (ANOVA or Kruskal Wallis, $P < 0.05$).

4.4.2 Immunological responses to Cd exposure

Neutral red uptake by haemocytes is shown in Table 4.3. Two-way ANOVA revealed some treatment effects at specific time points within individual treatments, but no overall combined effects of time and treatment (treatment × time, $P = 0.02$). Exposure of mussels to Cd resulted in a statistically significant increase in neutral red uptake by the haemocytes of animals from the 20 $\mu g\ l^{-1}$ treatment at days 4 and 8 (Kruskal Wallis, $P < 0.01$). Mussels from the 50 $\mu g\ l^{-1}$ Cd treatment did not show increases in neutral red uptake compared to controls (no significant treatment-effect), although neutral red uptake increased over time in the 20 $\mu g\ l^{-1}$ treatment.

Cd exposure had no statistically significant (ANOVA, $P > 0.05$) effect on phagocytosis activity of the haemocytes (Table 4.3). Only a small, but statistically significant time effect was found at day 8 in the 50 $\mu g\ l^{-1}$ Cd treatment compared to the animals at 4 days in the same treatment (ANOVA, $P = 0.003$). The cytotoxicity assay assessed the ability of the haemocytes from the mussels to lyse sheep erythrocytes. However, no statistically significant treatment effects were observed (Kruskal Wallis, $P = 0.2$). Cytotoxicty values on day 11 were 81.2 ± 0.9, 81 ± 0.7 and 82.5 ± 1.2 % for control, 20 and 50 $\mu g\ l^{-1}$ Cd, respectively. There were no treatment effects on total haemocyte counts (Kruskal Wallis, $P = 0.4$), and values remained in the normal range for all treatments (between 1.7 to 2.7 $x10^6$ cells ml^{-1}).

Table 4.3 Neutral red uptake and phagocytosis activity in haemocytes, and haemolymph electrolytes in *Mytilus edulis* from two separate experimental trials, Cd exposure alone, and Cd exposure after an LPS injection

Parameter	Cd Treatment ($\mu g\ l^{-1}$)	Time (days)			
		1	4	8	11
Cd alone trial					
Neutral red uptake (absorbance mg^{-1} protein)	control		0.56 ± 0.06	0.67 ± 0.05	
	20		1.53 ± 0.29 *	5.50 ± 0.26 *	
	50		0.46 ± 0.05	0.69 ± 0.18 #	
Phagocytosis activity ($x10^7$ particles mg^{-1} protein)	control		59.73 ± 2.75	89.21 ± 7.89	
	20		68.23 ± 3.32	81.64 ± 6.06	
	50		60.51 ± 4.19	78.96 ± 2.02 #	
Na (mM)	control		481.00 ± 8.86	472.50 ± 3.35	460.00 ± 2.04
	20		457.96 ± 26.13	462.00 ± 15.17 *	474.50 ± 8.39
	50		454.16 ± 4.90	462.00 ± 5.69	464.55 ± 3.44
K (mM)	control		11.51 ± 0.36	10.77 ± 0.22	10.04 ± 0.21
	20		11.38 ± 0.28	11.87 ± 0.43 *	11.35 ± 0.47 *
	50		9.99 ± 0.15 *	9.86 ± 0.09	10.84 ± 0.17 #
LPS + Cd trial					
Neutral red uptake (absorbance mg^{-1} protein)	Sham control	4.31 ± 0.71	1.02 ± 0.17 #		
	LPS	4.78 ± 0.21	1.21 ± 0.12 #		
	LPS + 20	4.72 ± 0.48	1.27 ± 0.09 #		
Phagocytosis activity ($x10^7$ particles mg^{-1} protein)	Sham control	72.13 ± 10.17	36.43 ± 4.19 #		
	LPS	92.06 ± 9.98	61.80 ± 13.75 *		
	LPS + 20	95.31 ± 12.90	102.69 ± 7.63 *		
Na (mM)	Sham control	452.50 ± 8.78	500.00 ± 3.53	531.25 ±12.64	
	LPS	482.00 ± 7.34	490.00 ± 10.41	492.00 ± 2.04 *	
	LPS + 20	496.00 ± 18.46	503.00 ± 19.63	481.00 ± 4.85 *#	
K (mM)	Sham control	7.33 ± 0.13	12.28 ± 0.32 #	11.68 ± 0.51	
	LPS	12.65 ± 0.29 *	12.77 ± 0.65	11.95 ± 0.42	
	LPS + 20	12.83 ± 0.42 *	12.81 ± 0.37	12.13 ± 0.33	

Data are means ± S.E.M., $n = 6$, per treatment at each time point. In the Cd alone trial animals were exposed to 20 or 50 μg l^{-1} Cd as $CdCl_2$ compared to a no-added Cd control. In the LPS + Cd trial, animals were given a LPS injection prior to exposure to 20 μg l^{-1} Cd. * indicates a significant difference from the control within each time point (ANOVA or Kruskal Wallis, $P < 0.05$). # indicates a significant difference from the previous time point within treatment ($P < 0.05$, ANOVA or Kruskal Wallis). Values for initial stock animals (time zero) at the start of the experiment were 0.96 ± 0.07, 61.06 ± 2.36, 483.3 ± 4.4 and 11.1 ± 0.3 for neutral red uptake, phagocytosis, Na and K, respectively in the Cd trial. Values for the initial stock animals in the LPS + Cd trial were 4.16 ± 1.05, 47.4 ± 2.10, 480.8 ± 23.9 and 10.1 ± 0.3 respectively.

4.4.3 Haemolymph chemistry during Cd exposure

Mussels were unfed during the experiment, and glucose levels were < 0.25 mM in all animals. Two-way ANOVA confirmed time-dependent increases in haemolymph glucose in the 20 and 50 μg l^{-1} Cd treatments ($P < 0.0001$), but not in the controls. There were no overall treatment-effects on glucose concentrations (two-way ANOVA, $P = 0.7$). Glucose values at the end of the trail were 0.23 ± 0.05, 0.23 ± 0.06 and 0.23 ± 0.05 mM for control, 20 and 50 μg l^{-1} Cd, respectively. Electrolyte composition of the haemolymph showed some small (Table 4.3), but statistically significant transient changes during the experiment, but there was no overall Cd-effect by the end of the exposure (ANOVA, $P > 0.05$). For example, there was a statistically significant increase in Na concentration in the 20 μg l^{-1} Cd treatment at day 8 compared to the control or the 50 μg l^{-1} Cd treatment (Kruskal Wallis, $P = 0.02$). For K, the treated mussels with 20 μg l^{-1} Cd showed significant increases above the control at days 8 and 11 days (Kruskal Wallis or ANOVA, $P = 0.03$ or less) (Table 4.3).

4.4.4 Histological alterations during Cd exposure

Gill pathology was absent in the control animals with no evidence of oedema or erosion of the gill filaments (Fig. 4.1). In the 20 μg l^{-1} Cd treatment, 2 out of 4 mussels showed evidence of necrosis and erosion of the frontal cilia on the gills. The effects of the 50 μg l^{-1} Cd treatment was more severe, with erosion of the frontal cilia on the gills in 5 out of 6 animals examined, and evidence of hypoplasia. The haemolymph vessel in each filament was also smaller suggesting the loss of haemolymph volume from the gills.

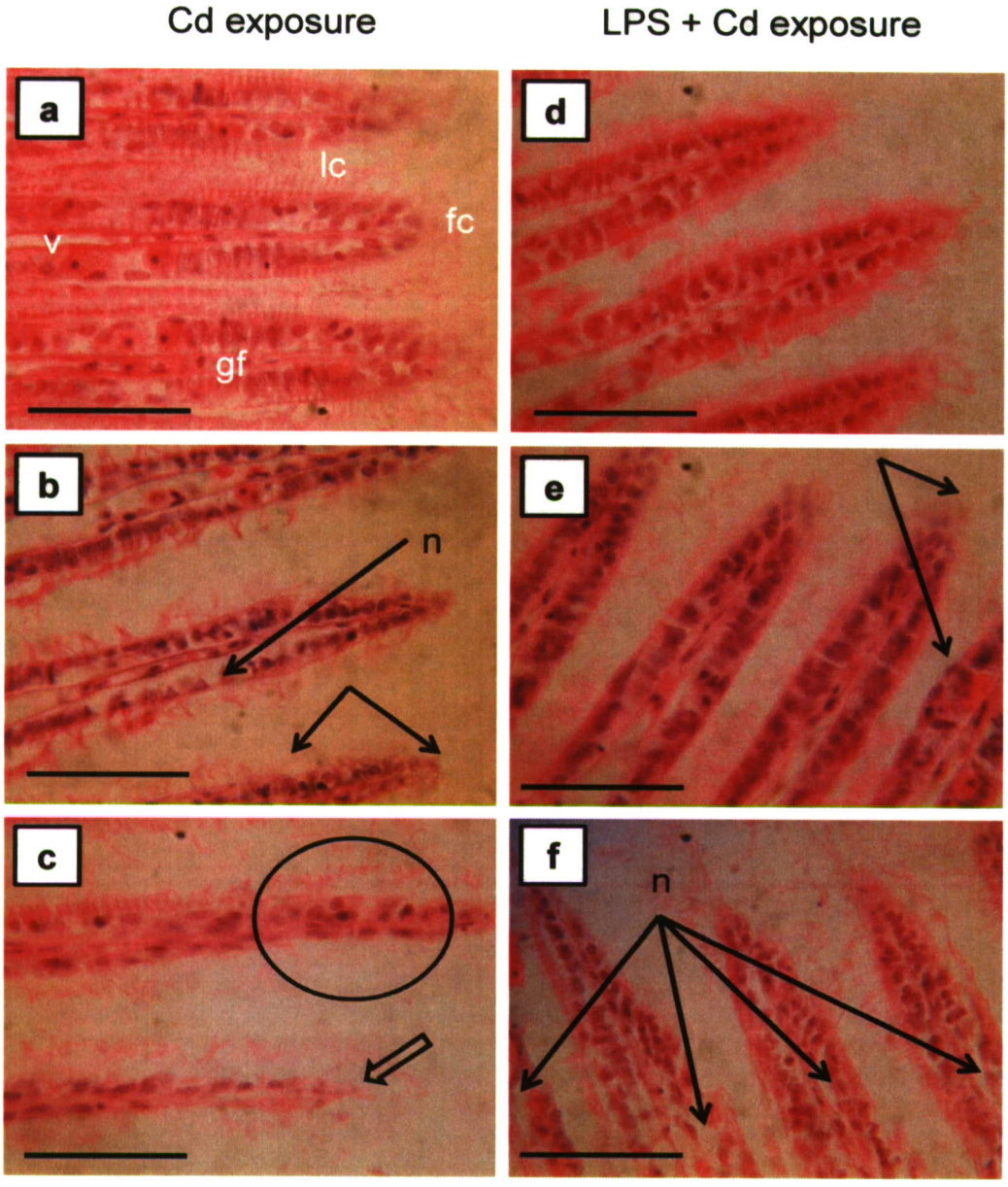

Fig. 4.1 Light micrographs of sections through gills of *M. edulis* showing histological structure of (a) control, (b) mussels treated with 20 µg l^{-1} Cd as $CdCl_2$, with a necrotic epithelium and less cilia (c) mussels treated with 50 µg l^{-1} Cd as $CdCl_2$, showing hyperplasia (circle) and cilia erosion (hollow arrow) (d) sham injected control, (e) mussels injected with LPS, with cilia erosion (arrow) and (f) mussels treated with LPS + 20 µg l^{-1} Cd as $CdCl_2$, showing necrotic gill filaments. Slides were stained with haematoxylin and eosin at 5-8 µm thickness. gf, gill filaments; fc, frontal cilia; lc, lateral cilia; v; haemolymph vessel; and n, necrosis. Scale bar, 50 µm.

The digestive gland of all the control animals showed normal architecture, with well defined digestive tubules and surrounding connective tissue. The epithelium of the intestine and the stomach appeared normal (Fig. 4.2). There were no treatment effects on the relative area of the digestive gland occupied by digestive tubules; % of tissue area as tubules was 65.8 ± 6.4, 62.2 ± 3.2, and 54.7 ± 7.9 for the control, 20 and 50 μg l^{-1} Cd treatment respectively. The thickness of the tubule wall was thinner in animals from the 20 μg l^{-1} Cd treatment (26.4 ± 0.6 μm) compared to the controls (45.3 ± 0.7 μm), or the 50 μg l^{-1} Cd group (40.3 ± 0.3). Cd exposure caused pathology in the digestive gland of all animals at the end of the experiment; especially in 20 μg l^{-1} Cd exposed group where 27 ± 0.3 % of the tubules examined showed injuries compared to only 8.6 ± 0.6 % in the control (statistically significant treatment effect, ANOVA, $P < 0.05$); and only 12.6 ± 1.2 in the 50 μg l^{-1} Cd (not different from controls). The percentage of tubules showing specific injuries such as necrosis (50 μg l^{-1} Cd, 6.7 ± 0.6; 20 μg l^{-1} Cd, 16.7 ± 0.3; control, 6.8 ± 0.6) and inflammation (50 μg l^{-1} Cd, 8.9 ± 0.7; 20 μg l^{-1} Cd, 8.7 ± 0.4; control 0 %, not observed) were also statistically higher in the 20 μg l^{-1} Cd group than the controls or 50 μg l^{-1} Cd treatment (ANOVA, $P < 0.05$). Pathology was evident in the stomachs of all animals exposed to the 20 μg l^{-1} Cd, although the pathologies varied in nature. One out of 6 animals showed cilia erosion, another 2 animals also exhibited connective tissue with fibrosis and inflammation (haemocyte infiltration).

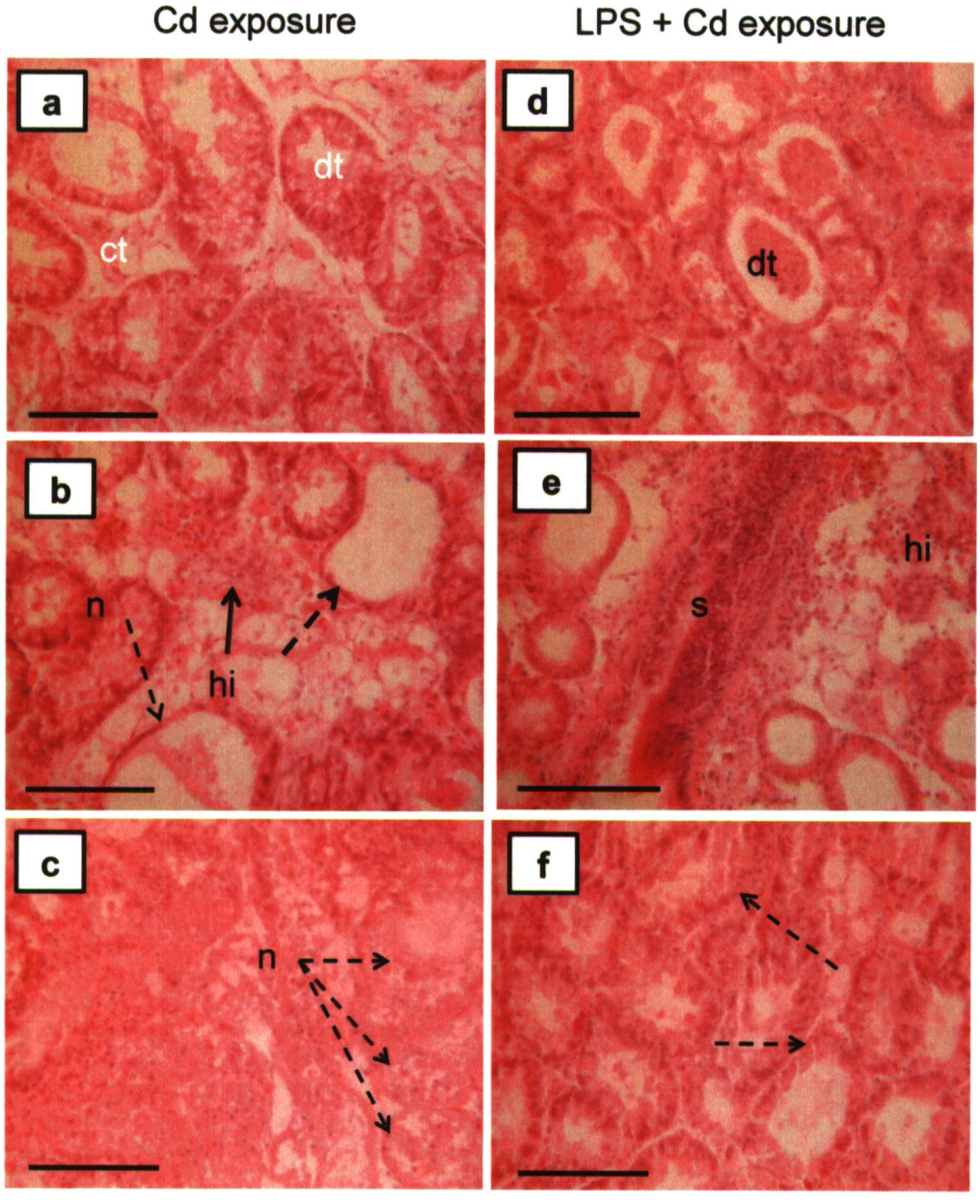

Fig. 4.2 Light micrographs of sections through digestive gland of *M. edulis* showing histological structure of (a) control, (b) mussels treated with 20 µg l^{-1} Cd as $CdCl_2$, with necrotic tubules and haemocytes infiltration, (c) mussels treated with 50 µg l^{-1} Cd as $CdCl_2$, showing necrosis of the tubules, (d) sham injected control, (e) mussels injected with LPS, showing inflammation of the connective tissue, and (f) mussels treated with LPS + 20 µg l^{-1} Cd as $CdCl_2$, with necrotic tubules. Slides were stained with haematoxylin and eosin at 5-8 µm thickness. ct, connective tissue; dt, digestive tubules; hi, haemocytes infiltration; s, stomach and n, necrosis (dashed arrows). Scale bar, 100 µm.

The posterior adductor muscle of the control animals showed normal histology with distinct muscle blocks containing intact bundles of muscle fibres. Connective tissue was well defined around each bundle of muscle fibres and the myocytes appeared normal. There was no evidence of oedema, necrosis or other injuries in the controls (Fig. 4.3). The adductor muscle of the mussels treated with 20 $\mu g\ l^{-1}$ Cd, showed loss of muscle fibre structural organization (1 animal), decreased extracellular spaces (2 animals), fissures (cracks) in the muscle bundles associated with the hydropic change, and hyperplasic muscle bundles also occurred in 3 out of other 4 animals examined. The 50 $\mu g\ l^{-1}$ treatment showed the same abnormalities as the 20 $\mu g\ l^{-1}$ Cd treatment, but more severe, with 2 animals showing loss of muscle fibre organisation, and another 3 animals showing decreased extracellular spaces between the bundles, although 2 animals appeared normal in this aspect.

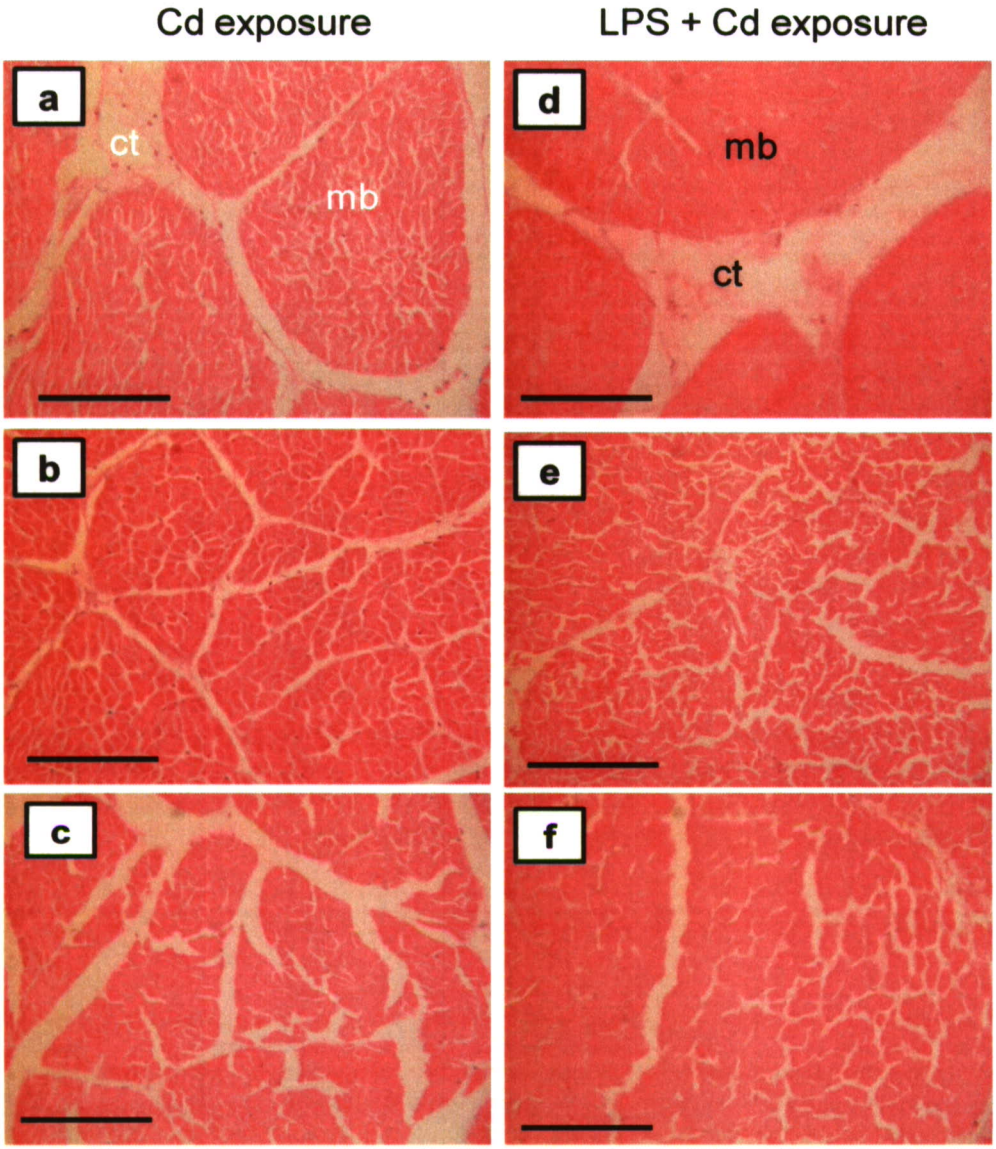

Fig. 4.3 Light micrographs of sections through adductor muscle of *M. edulis* showing histological structure of (a) control, (b) mussels treated with 20 µg l^{-1} Cd as $CdCl_2$, hyperplasic muscle bundles, (c) mussels treated with 50 µg l^{-1} Cd as $CdCl_2$, showing a decrease of extracellular space; (d) sham injected control, (e) LPS injected mussels with loss of muscle fibre integrity, and (f) mussels treated with LPS + 20 µg l^{-1} Cd as $CdCl_2$, showing fibrous structure disorganisation with fissures in the muscle bundles. Slides were stained with haematoxylin and eosin at 5-8 µm thickness. mb, muscle bundle; ct, connective tissue. Scale bar, 100 µm.

4.4.5 Cd accumulation and tissue electrolytes after LPS treatment

In the second series of experiments, mussels were exposed to 20 μg l^{-1} Cd with and without an injection of LPS to stimulate the immune system. There were no mortalities in the experiment, but animals in the LPS + Cd treatment showed statistically significant increases in Cd content compared to either the sham injected controls or LPS treatment alone (Kruskal Wallis, $P = 0.005$); with Cd content of the tissues increasing in the order digestive gland > gill > adductor muscle (Table 4.1). All three organs showed treatment and time interaction in Cd accumulation (two-way ANOVA, $P = 0.00001$). Cd concentrations in the gill and digestive gland, but not adductor muscle of animals from the LPS + Cd treatment, were lower than those in the Cd alone treatment from the first trial (Kruskal Wallis, $P < 0.02$ or less).

Na, K and Ca were measured in digestive gland, gills and adductor muscle on day 8. No significant differences in Na and Ca concentration recorded in the control and treated mussels with LPS alone or LPS + 20 μg l^{-1} Cd in all examined tissue (ANOVA, $P > 0.6$). However, K concentration of digestive gland showed a significant increase in the LPS + 20 μg l^{-1} Cd treated animals over the control and LPS alone (ANOVA, $P = 0.01$) (Table 4.4).

Table 4.4 Total Na, K and Ca concentrations in digestive gland, gills, and adductor muscle of *Mytilus edulis* after 8 days of exposure to 20 $\mu g l^{-1}$ Cd as $CdCl_2$ after pre-injection with LPS

Element ($\mu g\ g^{-1}$ dw)	Treatments	Digestive gland	Gills	Adductor muscle
Na	control	18.9 ± 1.1	44.2 ± 5.7	21.5 ± 1.6
	LPS	19.6 ± 1.4	44.5 ± 3.6	20.7 ± 1.1
	LPS+20 $\mu g\ l^{-1}$	20.2 ± 0.9	39.4 ± 2.6	21.0 ± 1.8
K	control	12.2 ± 0.5	13.4 ± 0.2	13.0 ± 0.6
	LPS	11.9 ± 0.9	13.8 ± 0.6	14.1 ± 1.7
	LPS+20 $\mu g\ l^{-1}$	15.2 ± 0.5 *$	14.5 ± 0.4	12.9 ± 0.2
Ca	control	6.3 ± 0.4	10.8 ± 0.3	5.6 ± 0.6
	LPS	6.1 ± 0.6	9.4 ± 0.8	8.1 ± 1.2
	LPS+20 $\mu g\ l^{-1}$	6.9 ± 0.5	8.9 ± 0.4	8.5 ± 1.5

Data are means ± S.E.M. $\mu g\ g^{-1}$ dry weight, n = 6 mussels per treatment. * indicates a significant difference from the control within each time point (ANOVA or Kruskal Wallis, $P < 0.05$). $ indicates a significant difference from the LPS treatment within each time point (ANOVA or Kruskal Wallis, $P < 0.05$).

4.4.6 Immunological responses to Cd after LPS treatment

Neutral red uptake by the haemocytes was unaffected by LPS injection or the LPS + Cd treatment (Table 4.3), although a significant time-dependent decrease in neutral red uptake was observed in all treatments (two-way ANOVA, $P = 0.00001$).

There was no overall combined effect of treatment × time (interaction) on phagocytic activity in all groups (two-way ANOVA, $P = 0.07$). However, there was a treatment-dependent effects on the phagocytic response at day 4 only (ANOVA, $P = 0.001$), and time-dependant decrease in the sham injected control group only (Kruskal Wallis, $P = 0.006$, see Table (4.3).

The cytotoxicity assay found no statistical differences in the ability of the haemocytes from the mussels to lyse sheep erythrocytes in the sham injected control compared to either LPS alone, or LPS + Cd (Kruskal Wallis, $P = 0.2$). Values of cytotoxic activity of haemocytes on day 8 were 90.7 ± 0.2, 92.0 ± 1.8 and 92.0 ± 0.5 for

the sham control, LPS, and LPS + 20 μg l^{-1} Cd, respectively. The total haemocytes count on day 8 increased in the LPS, and LPS + 20 μg l^{-1} Cd treated mussels, compared to the sham injected controls (Kruskal Wallis, $P = 0.01$). The mean values (x10^6 cells ml^{-1}) at day 8 were 2.2 ± 0.08, 3.9 ± 0.4, and 4.1 ± 0.7 for the control, LPS injected, and LPS + 20 μg l^{-1} $CdCl_2$ treatments respectively.

4.4.7 Haemolymph chemistry during Cd exposure after LPS treatment

Two-way ANOVA showed no treatment, time, or combined treatment x time effect on haemolymph glucose ($P > 0.5$). Glucose concentrations in the haemolymph from mussels at the end of the trail were 1.2 ± 0.1, 0.9 ± 0.2 and 1.1 ± 0.2 mM for sham controls, LPS, and LPS + Cd, respectively. Na concentration in haemolymph showed interaction between treatment and time (two-way ANOVA, $P = 0.006$). Treatment effects were observed in haemolymph Na concentration at day 8, where LPS and LPS + Cd treatments were significantly reduced compared to the control (Kruskal Wallis, $P = 0.006$). Also, K concentration in haemolymph showed interaction between treatment and time (two-way ANOVA, $P < 0.00001$). K showed a significant increase in values for the LPS, and LPS + Cd treatments, at 24 h compared to the sham injected control (ANOVA, $P = 0.005$). However, even these changes remained within the normal range of K and Na for mussels (Table 4.3).

4.4.8 SOD activity during Cd exposure after LPS treatment

SOD activity was measured in the extracellular fluid component of the haemolymph and in the haemocytes to estimate intracellular SOD activity. There were no treatment effects on extracellular SOD activity (ANOVA, $P = 0.5$). Values were 85.3 ± 13.5, 72.5 ± 16.7, and 94.9 ± 11.1 units ml^{-1} in controls, LPS, and LPS + Cd treatments, respectively at day 8. There were no overall significant time or treatment effects on

intracellular SOD activity (Kruskal Wallis, $P = 0.4$). Intracellular SOD activity on day 8 was 0.006 ± 0.001, 0.003 ± 0.001 and 0.006 ± 0.005 units $cell^{-1}$, for the control, LPS injected, and LPS + Cd treatments respectively.

4.4.9 Histological alterations during Cd exposure after LPS treatment

Gills from saline injected controls showed normal histology (Fig. 4.1). However, 4 out of 5 mussels injected with LPS showed cilia erosion of the gill filaments compared to the control (Fig. 4.1). Mussels from the LPS + Cd treatment showed more severe pathology, including necrotic gill filaments (Fig. 4.1).

Some mild histological abnormalities were found in the digestive gland of saline-injected controls. These abnormalities included slightly less well defined cell margins and the occasional small foci of damaged epithelial cells on some digestive tubules and the stomach (Fig. 4.2). All other structures were normal. All mussels injected with LPS showed alterations in the digestive gland, especially the digestive tubules which showed necrosis, diffuse and fragmented nuclei, and occasional foci where there was very poor distinction of the epithelial cells. One mussel from the LPS treatment showed some infiltration of haemocytes into the epithelium of stomach, and 2 out of 6 mussels showed pathology in the connective tissue (Fig. 4.2). Digestive gland from the LPS + Cd treatment generally showed similar pathology to the LPS-injected group, but more severe. In the LPS + Cd group, 5 out of 6 animals showed histological alterations in the digestive gland such that the epithelium was hard to distinguish. Digestive tubule epithelia showed frequent necrosis and foci with no clear distinction of epithelial cells (Fig. 4.2). Quantitative histological analysis of the digestive gland confirmed these observations on pathology, which occurred without changes in the percentage of digestive gland area as tubule (53.8 ± 14.9, 57.9 ± 6.5, and 42.8 ± 7.6 %) or width of the tubule wall (37.6 ± 0.4, 33.4 ± 0.1, 35.9 ± 0.3 μm), for the control, LPS,

and Cd + LPS treatments respectively. The effect of LPS + Cd was statistically greater than either the Sham or LPS injected groups (ANOVAs, $P = 0.02$) for the % of injured tubules (18.3 ± 0.3, 38.6 ± 0.9, 30.9 ± 1.4 %) and the % of tubules showing necrosis (16.5 ± 0.3, 29.6 ± 1.1, 53.6 ± 1.0 %), for the control, LPS, and Cd + LPS treatments respectively. However, there were no statistical differences in the incidence of inflammation in the tubules (11.1 ± 0.3, 26 ± 1.5, 9.8 ± 1.0 %), for the control, LPS, and Cd + LPS treatments respectively.

Saline injections, as expected, had some effects on the posterior adductor muscle, including some haemocytes infiltration (not shown), and decreased intracellular spaces between the myocytes at the site of injection (Fig. 4.3). However, all the muscles examined from the LPS injected animals showed some loss of muscle fibre integrity, cracking of the muscle bundle associated with hydropic change and decreased intracellular space (Fig. 4.3). In the LPS + Cd treatment the pathology was more severe, and all mussels exhibited hyperplasic muscle bundles, inflammation with loss of fibre integrity (Fig. 4.3).

4.5 Discussion

4.5.1 Effect of Cd exposure alone

Cd exposure was confirmed by Cd accumulation in the tissues (Table 4.1). Background levels were in the $\mu g\ g^{-1}$ dw range and were consistent with previous reports (e.g., about 3-7μg Cd g^{-1} dw, Roesijadi et al., 1984). Cd accumulation showed the expected exposure concentration response, with animals at the highest exposure concentration accumulating more Cd (Table 4.1). The pattern of Cd accumulation in the tissues showed that the digestive gland had higher Cd levels than the gills, and this is consistent with previous studies on bivalves (e.g., Roesijadi et al., 1984; Zorita et al., 2007). There were limited effects of Cd exposure on the haemolymph chemistry. At the end of the

experiment both Na and K were in the normal range expected for this species (Na, 454 - 583; K, 9.9 -11.9 mM; consistent with Potts (1954). The low but stable haemolymph glucose levels were also consistent with using unfed animals during the experiment. Overall, the haemolymph chemistry indicates a normal extracellular environment for the haemocytes without osmotic stress.

Neutral red retention by haemocytes is an indicator of the integrity of the lysosomal system in the cell, and therefore of cell health (e.g., Lowe et al., 1995). Neutral red uptake by haemocytes increased significantly at day 4 and 8 of exposure in the 20 $\mu g\ l^{-1}$ Cd treatment. Coles et al. (1995) made similar observations *in vivo* at a much higher Cd concentration (400 $\mu g\ l^{-1}$). However, the neutral red response did not increase linearly with the external Cd concentration (Table 4.3), despite large increases in the Cd content of the tissues (Table 4.1). The reason for this is not clear, but Butler and Roesijadi (2001) also found decreased neutral red retention with increasing $CdCl_2$ levels up to 100 μM for a 20 h exposure in oysters.

Exposure to 20 or 50 $\mu g\ l^{-1}$ Cd for 11 days had no clear effect on the ability of haemocytes to phagocytise zymozan particles (Table 4.3). In shorter experiments, Coles et al. (1995) also found no effect of 40 or 400 $\mu g\ l^{-1}$ Cd on the phagocytosis activity of haemocytes from *M. edulis* after 7 days. Similarly, haemocytes from the clam *Tapes philippinarum* were also unaffected by 150- 450 $\mu g\ l^{-1}$ Cd exposure for 7 days (Matozzo et al., 2001).

The ability of haemocytes to lyse sheep red blood cells (cytotoxicity assay) also showed no clear Cd-dependent effect, and cell counts in the haemolymph did not change. Taken together, the normal haematology, the lack of change in the phagocytosis assay, and the limited effects on neutral red uptake suggests that Cd exposure alone does not damage the immune functions of the haemocytes in our experimental conditions. Despite this apparent "no observed effect" on immune functions of

haemocytes for the parameters measured here, some pathologies occurred in the tissues (see below).

All the organs showed histological change associated with Cd exposure (Figs. 4.1, 4.2 and 4.3). Cd exposure caused loss of the lateral cilia and changes in branchial haemolymph vessels in the gills. Similar effects are well known in fish (e.g., Mallatt 1985), and have been reported in bivalves (e.g., Fasulo et al., 2008). The loss of the lateral cilia would likely compromise filter feeding. The gills are also an osmoregulatory organ, but the haemolymph chemistry suggests that osmoregulation was still effective; albeit the animals were in normal seawater and not osmotically challenged.

Necrosis, inflammation and some disruption of the intestinal and stomach epithelium of the digestive glands during Cd exposure was also observed (Fig. 4.2). Similarly, inflammation and necrosis in the digestive tubules has been reported in *Mytilus* sp. after 30 days of exposure to 50 $\mu g\ l^{-1}$ Cd (Da Ros et al., 1995); and also for *M. edulis* exposed to 50 $\mu g\ l^{-1}$ $HgCl_2$ for 11 days (Sheir et al., 2010). It is unclear whether the level of injury we observed would compromise digestive functions, but the necrosis and inflammation in the digestive gland was associated with Cd elevation in the tissue (Table 4.1). We also observed injury to the posterior adductor muscle during Cd exposure, characterised by loss of muscle fibre bundle integrity and some inflammation (Fig. 4.2). The adductor muscles are responsible for closing the shell, and so damage to this muscle may compromise feeding behaviour, or alter ventilation.

4.5.2 Effect of LPS pre-treatment prior to Cd exposure

Injection of LPS alone compared to the sham saline injection had no effects of the neutral red uptake, or haemolymph glucose levels. Similarly, Hernorth (2003a) found no effect of LPS on the viability of haemocytes from *M. edulis*. There were some histological changes in the gills (necrosis, Fig. 4.1), and injuries in the digestive gland (Fig. 4.2), with evidence of haemocyte infiltration; indicating an immune response following LPS injection alone. This observation is consistent with the sensitivity of the mammalian gut epithelium to LPS (Ismail and Hooper, 2005), and similar recent observations on digestive gland in *M. edulis* prior to Hg exposure (Sheir et al., 2010). There were also some changes in the adductor muscle (Fig. 4.3) consistent with a mild inflammation. LPS injections into adductor muscle are known to cause local inflammation at the injection site (Sheir et al., 2010), so this is to be expected.

One hypothesis for the interaction of LPS with Cd exposure is that the pre-injection has an immune effect on Cd uptake or toxicity. The general pattern of Cd accumulation with Cd alone compared to LPS + Cd was similar, but the Cd levels were lower in the LPS + Cd treated animals (Table 4.1); suggesting that LPS pre-injection delayed Cd uptake. Despite this, the pathology in the LPS + Cd treatment was generally similar or worse than LPS alone (Figs. 4.1, 4.2 and 4.3). However, there were no additional effects of Cd following LPS treatment on neutral red uptake, or the cytotoxicity of haemocytes, or haemolymph chemistry. There was an increase in the phagocytosis ability of haemocytes at day 8 in the LPS + Cd treatment (Table 4.3), suggesting at least some response of the cells. Brousseau et al., (1999) also found some stimulation by $CdCl_2$ *in vitro* using haemocytes from the clam *Mya arenaria*, but here we show a small, but additional effect of LPS. However, overall, the measurements on haemocytes suggest that LPS pre-treatment does not prime the cell's immune response to Cd very much. Sheir et al. (2010) made very similar observations with the same LPS

injections followed by exposure to 50 μg l^{-1} $HgCl_2$ for 8 days, where the animals showed evidence of histological change consistent with an immune response; but the haemocytes and haemolymph remained functionally normal. The injury to the digestive gland observed here with LPS + Cd (Fig. 4.2) was also noted during exposure to 50 μg l^{-1} Hg as $HgCl_2$ for 11 days after LPS injections, in very similar experimental conditions (Sheir et al., 2010). This suggests that some of these effects may not be metal-specific, but for example, could be related to common aspects of metal bioreactivity. For instance, both metals bind avidly to thiol groups on proteins, and this is a well known mechanism for toxicity and subsequent oxidative stress (review, Stohs and Bagchi 1995; e.g., for haemocytes, Gomez-Mendikute and Cajaraville 2003). However, haemocytes in this experiment did not show a depletion of intracellular SOD activity, and extracellular SOD activity was also unchanged; suggesting this was not a significant factor for these cells in our experimental conditions. This is also consistent with modest changes in the mitochondrial volume and membrane potential in haemocytes from oxysters exposed to Cd (Sokolova et al., 2004).

4.6 Conclusion

M. edulis accumulates Cd in the gills, digestive gland, and adductor muscle. This Cd accumulation is associated with inflammation and organ pathology, but the physiological integrity of the haemolymph and haemocytes appeared to be intact. The haemocytes showed no evidence of immuno-stimulation or suppression during Cd exposure alone. An LPS pre-treatment exacerbated tissue injury in the gill, digestive gland, and adductor muscle associated with Cd exposure; suggesting that the haemocytes were either unable to respond, or were not signalled to respond to the stress (e.g., via caspase, Sokolava et al., 2004) in our experimental conditions. Further work, especially on cell signalling is needed to understand the combined effects of LPS + Cd on haemocytes.

Chapter 5

Immunological responses and histopathology of the marine mussel (*Mytilus edulis*) from clean and polluted sites in South West England

Chapter 5

Immunological responses and histopathology of the marine mussel (*Mytilus edulis*) from clean and polluted sites in South West England

Abstract

Field collected mussels are widely used for laboratory experiments on metal uptake, and as a biomonitoring tool to assess the effects of pollution on ecosystems. The aim of this study was to determine the immunological status and organ pathologies in animals from a clean reference site and a contaminated ecosystem in South West England to determine whether these end points could be useful in biomonitoring. The study also provides baseline data for further laboratory exposures (Chapter 6) on the animals. *Mytilus edulis* collected from both sites were analysed for trace metals (Cd, Cu, Fe, Pb and Zn) in the tissues and haemolymph. Then the immune functions of haemocytes (neutral red uptake and phagocytosis), haemolymph and tissue biochemistry, condition index and morphometric analysis were assessed. Organ histology was examined in the digestive gland, gills, and male and female gonads from both sites. Cd and Cu levels were significantly higher (4 and 1.9 fold increase, respectively) in gonads from animals at the polluted site compared to the reference site (Student *t*-test, $P < 0.05$). Mussels from the contaminated site also showed higher Fe and Zn levels in the gills (e.g., Fe and Zn concentrations, 534.2 ± 67.5 and 57.7, Mann-Whitney, $P < 0.05$). Good correlations were also found between shell length and Zn, Fe, Cd or Pb, but not for other metals. Neutral red uptake demonstrated significant increase (45.5 %) in the polluted site, but not the phagocytosis. Morphometric measurements showed that the shells of mussels from the polluted site were more fragile than those from the reference site. The Condition index was lower in mussels from the polluted site compared to the reference site. Necrosis, inflammation and atrophy are the common features of polluted site effects on the mussel's tissues which devoid from the clean site. The data show that

metal pollution modulates the immune and other physiological functions of mussels, and that organ histopathology is a sensitive method to differentiate clean and contaminated sites.

5.1 Introduction

Urban and industrial areas on the coastline inevitably introduce contaminated effluents into marine and estuarine ecosystems. Indeed, the majority of the UK's heavy industry is located where effluents can be easily discharged into estuaries. This anthropogenic pollution can result in the accumulation of metals in marine sediments, and then into the organisms in the marine food chain, including shellfish (Dallinger et al., 1987; Stewart et al., 2004; Rainbow, 2002). In addition to chemical monitoring (Garrigues et al., 1993), biomonitoring is also a useful tool to assess ecosystem health (Au, 2004). *Mytilus edulis* is a sedentary organism that is easily collected from the shore, and has been extensively used in monitoring programmes (e.g., Mussel Watch Program worldwide, Cantillo, 1988). The main purpose of biomonitoring is to detect change in the ecosystem, or the health of the organism that lives in it. One set of tools used in biomonitoring are biochemical biomarkers. A biomarker is defined as a biological response to a chemical(s) that gives a measure of exposure, and/or biological effect (Peakall, 1994; Handy et al., 2002; Handy et al., 2003; Depledge, 1994). Biomarkers are therefore used to not only measure changes in the health of the organisms, but to detect different categories of pollutants (Handy et al., 2002). Traditionally, biomarkers were mainly biochemical responses (e.g., changes in enzyme activities, metal accumulation in the tissues), but now also include a range of molecular responses including DNA damage assays (COMET assay, Canty et al., 2009) and more recently the use of genomics to measure changes in patterns of gene expression. However, immunological responses have also been used (Galloway and Depledge, 2001), and histology is also a

powerful tool for monitoring pollution (Au, 2004; Hinton et al., 1992; Handy et al., 2002)

However, linking adverse effects in organisms with specific pollution events is very challenging. The cause and effect paradigm is central to the application of acceptable biomarkers (Peakall, 1994). Field work is always compounded by complex and dynamic changes in the natural environment. These external factors can be large scale changes in biogeochemistry such as the thermal effect of the atmosphere (climate change), rainfall and rates of deposition into river catchments, and the varying hydro-biological characteristics of the estuarine environment itself relating to long term or season change (Ng et al., 1996; Milchakova and Philips, 2003; Turner and Millward, 2000). There are also short term changes in chemistry (e.g., with tidal cycles) and biomarkers may need to work against a background of change in abiotic factors such as temperature, salinity and dissolved oxygen (Handy et al., 2003). Also, biotic factors like genotype, phenotype, inherent sensitivity of the species, sex, age and body size may also alter biomarker responses (Peakall, 1994; Handy et al., 2003). It is also important to recognise that pollution controls may need to be site-specific and it is important to choose an organism which show spatial and temporal changes in metal accumulation (Rainbow et al., 2000), or other biomarker responses (Handy et al., 2003).

The toxic effects of pollution depend mainly on the type and concentration of pollutants. In most cases in the field, concentrations are low (e.g., μg/l exposures of metals) that cause subtle sublethal effects that may lead to ill health or chronic diseases (Stewart et al., 2004). Furthermore, the toxicity of complex mixture varies both temporally and spatially from their sources (Peakall, 1994). Of course, industrial accidents may still cause acute pollution events that lead to the death of the organisms (Eisler, 1987). The challenge in the field is to detect a biomarker response for a specific

event or time period against all the abiotic and biotic factors above, and with often a long history of low level pollution at contaminated sites. Clearly, the time, expertise and cost needed to assess the full impacts of pollution on the environment are rarely achieved in the real world (Galloway et al., 2002). However, the difficult problem of comparing the polluted organisms to control ones can be made easier by carefully choosing reference sites which have similar general hydrology and geochemistry to the polluted locations (Handy et al., 2003). The use of historic records or previous monitoring data can also be helpful in interpreting biomarker responses. A lack of data on the normal state of the population before pollution occurs often prevents an accurate impact assessment (Depledge and Fossi, 1994).

Mussels have been used in several field studies on environmental contamination from metals. For example, trace metal concentrations (Cd, Cu, Hg and Pb) were measured in tissues from mussels in Lake Balaton from 1996-2001, and compared with data obtained between 1978 and 1980 at the same location and found the level of Cd and Hg concentrations increased, while Pb contamination decreased during the past 20 years in mussels (Salánki et al., 2003). Svärdh and Johannesson (2002) used haemocyte counts and organ pathology in *M. edulis* during biomonitoring of an impacted area with industrial plants (South Skagerrak, Mexico). Lowe and Fassato (2000) found correlation between contaminant body burdens and two lysosomal acid hydrolases of digestive gland in *M. galloprovincialis* from a contaminated site in Venice Lagoon, Italy. Bayne and Moore (1998) recommended cellular immune defences in aquatic invertebrates as indicators of aquatic pollution and risk assessment. Developing granulocytomas in interstitial tissue, necrosis and atrophy in *M. edulis* tissue as a result of chemical contamination (metals and organic xenobiotics of industrial origin) in the field was found by Auffret (1988). Condition index and lysosomal stability were also used as biomarkers for anthropogenic pollutants on the ribbed mussel, *Guekensia demmissa*

(Galloway et al., 2002). Biomonitoring studies have been done on polluted sites of the Black Sea (Romania) using *Mytilus* sp. (Roméo et al., 2005) and South West UK by Turner et al. (2009).

This study aimed to use mussels from two contrasting locations. Plymouth Sound, Plymouth, is a busy water way and major port in South-West England. The water and sediment contains metal inputs from a variety of sources including antifouling paint where the main biocidal component is Cu (~30 % w/w) and Zn (~10 % w/w) (Gammon et al., 2009). Plymouth Sound and the estuaries flowing into it have been extensively studied for metal chemistry (e.g., Challenger et al., 1993; Turner et al., 2009). The objective of this work was to compare the cellular immune responses, haemolymph electrolytes and tissue metals, and organ pathologies in mussels from the polluted and reference sites. Particular attention was given to the metals found in Plymouth Sound, including biologically essential metals (Cu, Fe, and Zn) and non essential metals such as Cd and Pb.

5.2Materials and methods

5.2.1Field sites and mussel collection

M. edulis mussels were collected from the harbour area as an impacted area of industry and shipping effluents (antifouling paint). In contrast, a clean or reference area was chosen as a control at Port Quin, Cornwall which is a small village with no significant industrial inputs. The Mount Batten area of Plymouth Sound, Devon, UK (grid reference SX483532), was chosen as a polluted site (Fig. 5.1). The Mount Batten marina is a mixture of commercial and pleasure craft, and with significant historic use of the piers by the Royal Navy. Samples were collected on a small area of mud flat next

to the public slipway. This area currently has several working ship yards immediately adjacent to the beach, and the beach itself has been used for storing and welding large metal superstructures (mooring piles, pontoons, parts of off-shore platforms, and small merchant vessels). The site will therefore have both direct and diffuse metal inputs. The reference site was Port Quin, North Cornwall, UK (grid reference SW971806). More than 100 hundred animals were collected at each site for experiments (see Chapter 6), but 9 mussels were analysed for both sites immediately on sampling during 5th May 2009. Mussels were collected carefully by using scissors to cut the byssal threads that attached them to the wood and mud and immediately transferred to a cool box containing sea water. Individuals from each site were taken to the laboratory in tanks filled with water from their collection sites and then kept in aquaria with constant aerations for a few hours until all the samples could be processed. The journey time from Plymouth Sound to the laboratory is only 20 minutes, and from Port Quin about 2 hours. The animals were kept cool and aerated to minimise transportation stress. Morphometric characters as shell length, weight, total body and flesh weight were measured for each sample from both sites. Shell length and total body weight for the reference site were 4.8 ± 0.1 and 14.9 ± 1.2, respectively and shell length and total body weight for the polluted site were 5.8 ± 0.2 and 16.6 ± 1.6, respectively. The seawater of Plymouth Sound is ~ 33 salinity and ~ 7.5 pH (Turner et al., 2009).

Haemolymph chemistry, haemocyte counts, and tissue collection, immunological assays including neutral red and phagocytosis, condition index, and histology and histological measurements were carried out on each animal from each site (see Chapter 2 for details of the methods).

Fig. 5.1 Mount Batten site
a: United Kingdom, Devon, Plymouth (circle)
b: Plymouth Sound, Mount Batten (circle)
c: Mount Batten sampling site

5.2.2 Metal analysis

Metal analysis in tissues was performed to confirm tissue burdens of metals in mussels from both sites using the tissue digestion method and analytical procedure described in Chapter 2. Sediment samples were collected to confirm the metal contamination at the exact location where mussels were taken. For sediment analysis, sediment sample from Mount Batten was collected from the surface layer (0 – 8 cm depth) using an 8 cm tube and kept in the refrigerator. The processing and analysis of sediments were based on the protocol of Turner et al. (2009). Each sediment sample was oven-dried to a constant weight (50 °C for 2 days). The dried sediment was then sieved through a 2 mm mesh to remove stones and gravels. Then it is ground in a mortar and passed through a 180 μm sieve to collect the fine dust. The dust was then digested in a Pyrex boiling beakers with a mixture of 3:1 concentrated hydrochloric acid: concentrated nitric acid, respectively for 1 h in a 100 °C hot – block along with an empty tube as a procedural blank. Then samples were allowed to cool. The cooled digests were transferred to a clean (acid washed) 25 ml volumetric flask and then diluted to mark with 2 % nitric acid made up in Milli-Q Water. Metals (Cd, Cu, Fe, Zn, Pb, Na, K, and Ca) were analysed in sea water and sediment samples collected from the reference and polluted sites (Table 5.1).

Table 5.1 Concentrations of metals in water and sediment collected from the reference and polluted sites

Sample	Site	Cd	Cu	Fe	Zn	Pb	Na	K	Ca
Sea water	Reference	< 0.007	< 0.006	0.04	0.005	0.04	5646.8	34.5	340.87
	Polluted	< 0.007	< 0.006	0.6	0.01	< 0.03	6582.9	35.7	326.76
Sediment	Reference	0.5	9.1	7484.8	19.9	12.9	1845.5	11.8	160656.51
	Polluted	115.7	44551.9	5750281.2	39831.0	54752.8	591505.2	16761.8	4234093.75

Data are $\mu g\ g^{-1}$ dry weight for sediment and $\mu g\ ml^{-1}$ for sea water. Note. Procedural detection limit for sea water samples was 0.007, 0.006 and 0.03 $\mu g\ ml^{-1}$ for Cd, Cu and Pb, respectively.

5.2.3 Condition index

The condition index (CI) was calculated according to Aguirre (1979) from the following formula: CI = [MW/ (TW- SW)] x 100

Where MW; wet meat weight, TW; total wet weight and SW; shell weight.

5.2.4 Statistical analysis

In order to explore significant difference between reference and polluted sites, data sets were analysed using Statgraphics v5.1 software (StatSoft, USA). Data are expressed as means ± S.E.M. for n = 9 mussels per site. Data were analysed using unpaired two sample comparisons to compare the difference of pre-exposed mussels of the polluted site to clean site mussels. When Student t-test is not applied; otherwise, Mann-Whitney test was applied to compare medians. Correlations between variables were tested by Pearson's correlation coefficient. Plotting the correlation, r^2, best fit curve and equation were carried out by SigmaPlot 11.0 software.

5.3 Results

5.3.1 Metal accumulation and osmoregulation

Metals were analysed in digestive gland, gills, gonads and haemolymph of mussels collected from the reference and polluted sites (Table 5.2). Cd and Cu levels were significantly higher (300 and 88 % increase, respectively) in the gonads of animals from the polluted site compared with the reference site ones (Student *t*-test, $P < 0.05$) and haemolymph Cu (Student *t*-test, $P < 0.01$). The digestive gland and gills of mussels from the polluted site showed an increase in Fe and Zn levels when compared to the reference site (Mann-Whitney, $P \leq 0.05$). Fe in haemolymph decreased by 50 % in polluted mussels compared with reference site mussels (Student *t*-test, $P = 0.0003$). The digestive gland and gonad of animals from the polluted site showed increased Pb concentrations (Student *t*-test or Mann-Whitney, $P < 0.01$), but the haemolymph and gills did not (Table 5.2).

The haemolymph and digestive gland of mussels collected from the polluted site were high in Na (Student *t*-test, $P \leq 0.02$) but not the gills or gonads. K followed the same pattern of Na in all three tissues (Table 5.2, Student *t*-test, $P = 0.0001$), but decreased in haemolymph (Student *t*-test, $P = 0.005$). Ca decreased in gills and digestive gland and significantly in haemolymph (Student *t*-test, $P = 0.0001$), but increased in gonads of polluted site mussels (Table 5.2).

Table 5.2 Metal concentrations in digestive gland, gills, gonads and haemolymph of *M. edulis* collected from the reference and polluted sites

Metal	Site	Digestive gland	Gills	Gonads	Haemolymph
Cd	Reference	0.7 ± 0.1	0.9 ± 0.4	0.1 ± 0.02	0.02 ± 0.0
	Polluted	0.9 ± 0.2	0.6 ± 0.1	0.4 ± 0.1 *	0.01 ± 0.0
Cu	Reference	6.5 ± 0.3	4.7 ± 0.7	1.7 ± 0.4	0.03 ± 0.1
	Polluted	8.3 ± 0.9	4.6 ± 0.3	3.2 ± 0.6 *	0.1 ± 0.02 *
Fe	Reference	73.6 ± 5.2	108.1 ± 8.1	58.1 ± 7.7	0.2 ± 0.01
	Polluted	269.6 ± 27.5 *	534.2 ± 67.5 *	104.2 ± 20.8	0.1 ± 0.01 *
Zn	Reference	144.5 ± 35.2	32.3 ± 1.8	45.5 ± 10.8	0.2 ± 0.03
	Polluted	50.1 ± 2.9	57.7 ± 10.9 *	30.7 ± 8.1	0.1 ± 0.01
Pb	Reference	1.7 ± 0.2	2.7 ± 1.2	0.5 ± 0.1	0.2 ± 0.03
	Polluted	5.6 ± 0.9 *	5.9 ± 1.0	2.2 ± 0.5 *	0.3 ± 0.1
Na	Reference	245.4 ± 117.7	10696.9 ± 225.9	3315.8 ± 396.0	463.3 ± 5.3
	Polluted	3651.5 ± 257.0 *	1078.0 ± 459.2	4598.3 ± 500.0	477.8 ± 2.8 *
K	Reference	553.1 ± 13.0	726.1 ± 4.2	719.6 ± 27.8	12.1 ± 0.2
	Polluted	698.3 ± 21.5 *	719.3 ± 10.8	766.9 ± 29.7	11.2 ± 0.2 *
Ca	Reference	753.4 ± 18.3	1603.7 ± 71.0	532.6 ± 40.8	303.8 ± 2.4
	Polluted	597.6 ± 64.9	1436.2 ± 83.0	590.9 ± 44.7	270.1 ± 2.6 *

Data are means ± S.E.M. $\mu g\ g^{-1}$ dry weight for tissue, $\mu g\ ml^{-1}$ for haemolymph, $n = 9$ mussels per site. * indicates a significant difference from the reference site within each tissue (Student *t*-test or Mann-Whitney, $P < 0.05$). Note. The reference site is Port Quin (Cornwall) and the polluted site is Mount Batten (Plymouth).

There were relatively strong correlations between metals like Cd and shell length (Pearson's, $P = 0.0001$), and between Pb and shell length (Pearson's, $P = 0.01$) (Fig. 5.2). Furthermore, there was also a relatively strong correlation between the essential metals like Zn and shell length (Pearson's, $P = 0.01$), and between Fe and shell length (Pearson's, $P = 0.0001$), but not between Cu and shell length (Pearson's, $P > 0.05$) (Fig. 5.3). Only weak correlations were found between Ca concentration and shell length, or Ca and Cd or Pb concentrations (Pearson's, $P > 0.05$) (Fig. 5.4).

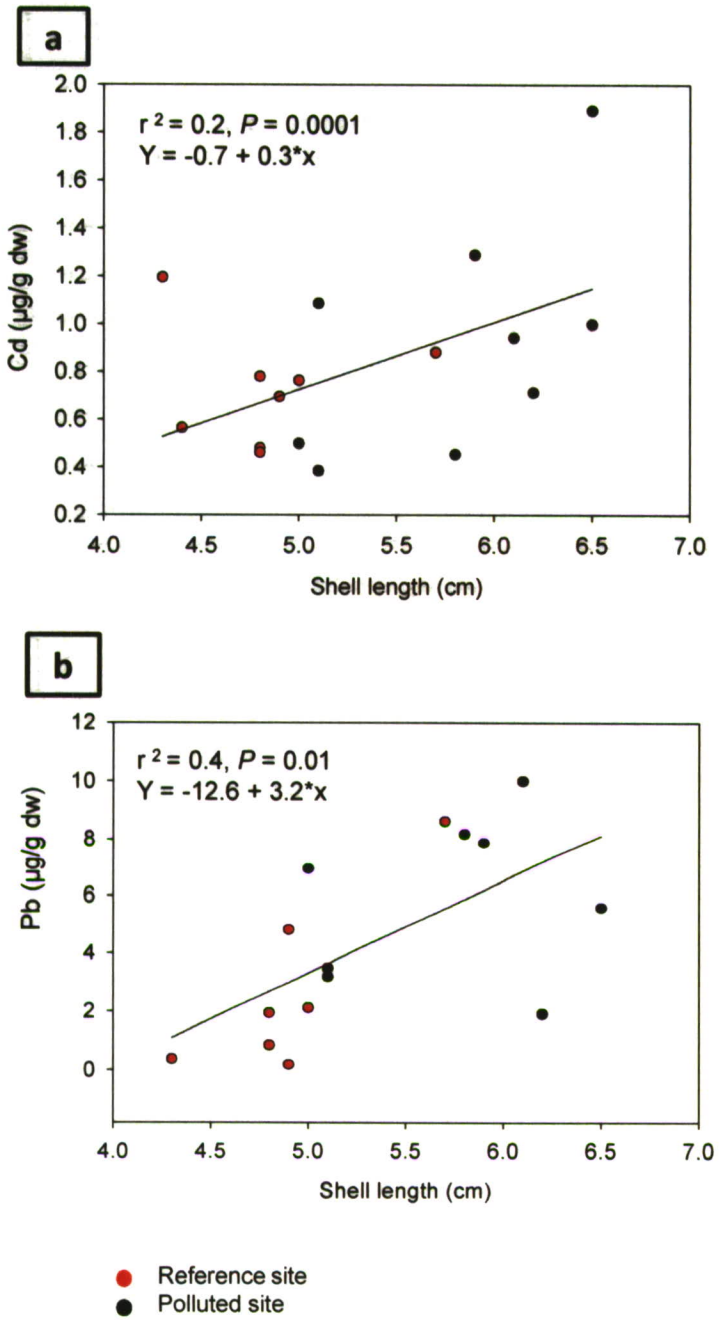

Fig. 5.2 Regression analysis illustrating correlations between shell length and (a) Cd concentrations (Linear fit); (b) Pb concentrations (Linear fit); of mussels collected from the reference and polluted sites, n = 7 - 9.

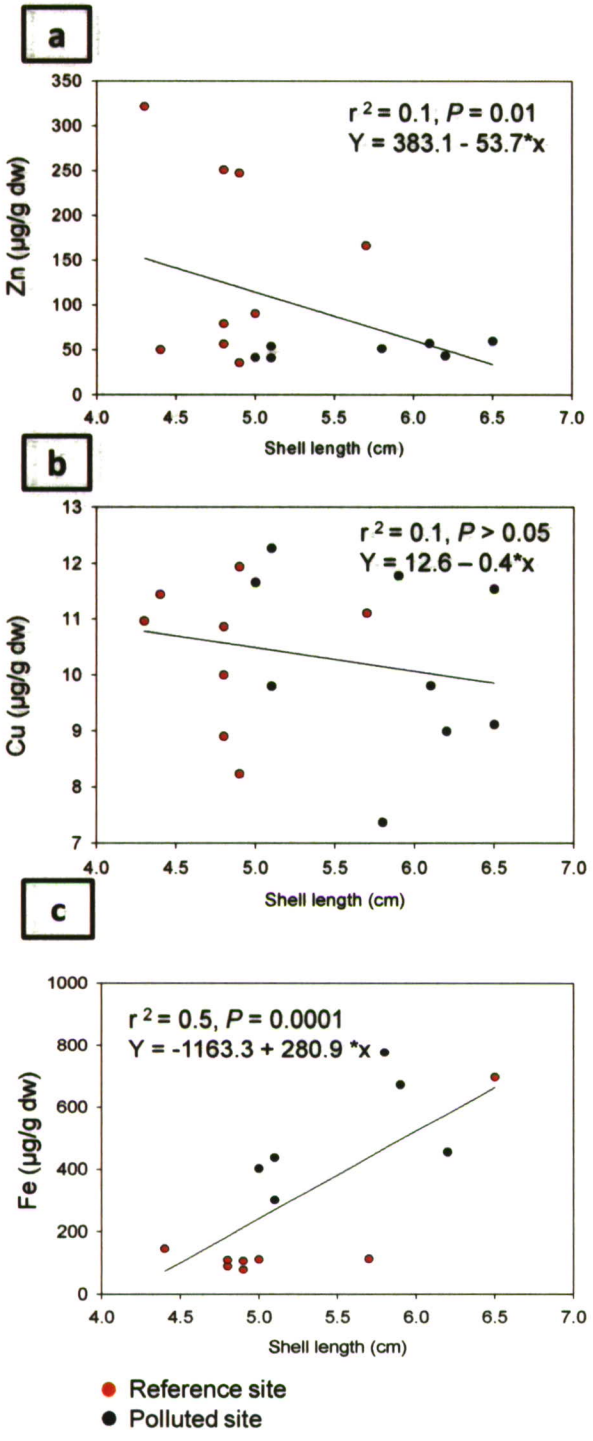

Fig. 5.3 Regression analysis illustrating correlations between shell length and (a) Zn concentrations (Linear fit); (b) Cu concentrations (Linear fit); and (c) Fe concentrations (Power fit) of mussels collected from the reference and polluted sites, $n = 7 - 9$.

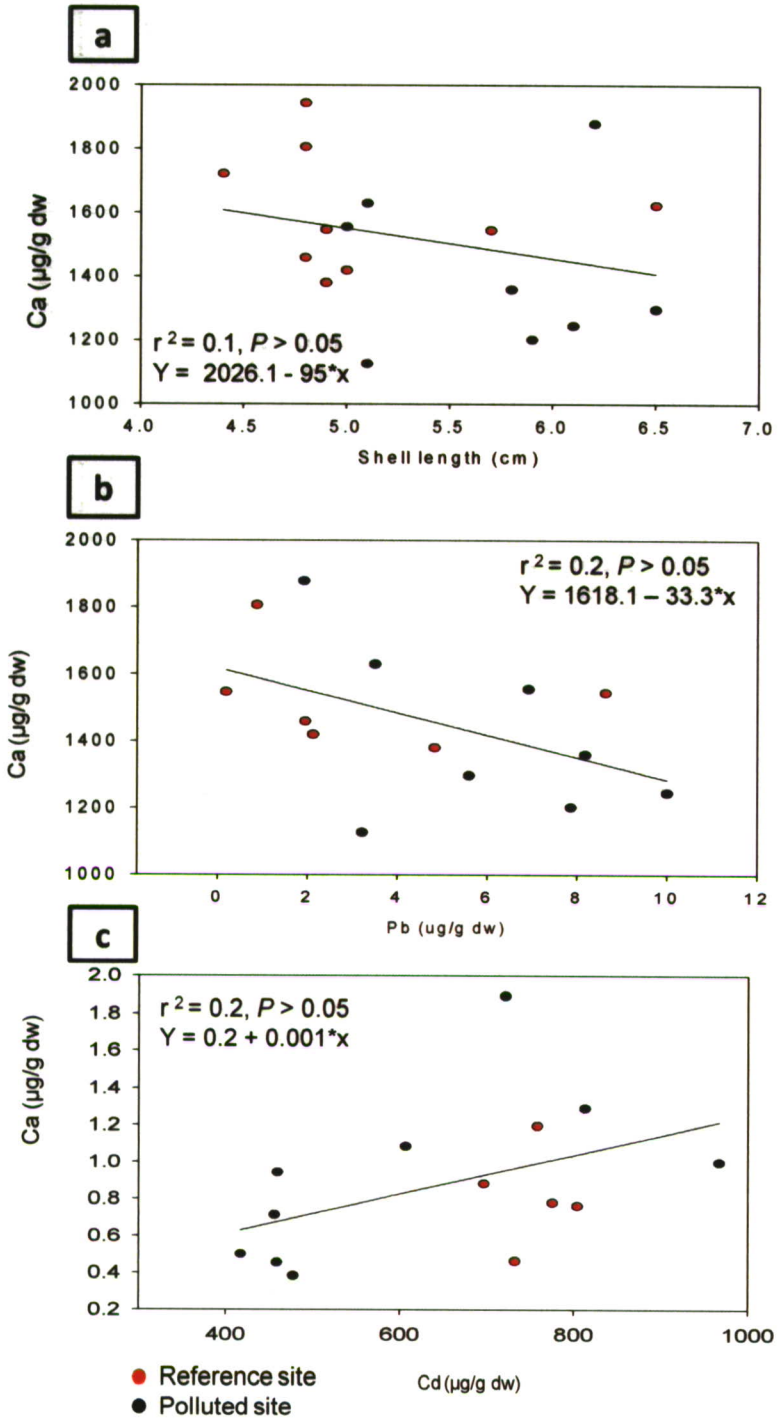

Fig. 5.4 Regression analysis illustrating correlations of Ca concentration and (a) shell length (Linear fit); (b) Pb concentrations (Linear fit); and (c) Cd concentrations (Linear fit); between mussels collected from the reference and polluted sites, n = 5 - 9.

5.3.2 Immunological responses

Neutral red uptake and phagocytic activity were assessed in haemocytes from the reference and polluted sites (Table. 5.3). Neutral red uptake increased in haemocytes from animals collected from the polluted site, compared to the reference ones (Student *t*-test, $P = 0.02$). However, phagocytosis activity was inhibited in the haemocytes of the polluted mussels when compared to reference mussels (Student *t*-test, $P = 0.5$).

Table 5.3 Neutral red uptake and phagocytosis activity (measured at 550 nm) in haemocytes of *M. edulis* collected from the reference and polluted sites

Parameter	Site	Mean ± S.E.M.
Neutral red uptake (Absorbance units mg^{-1} protein)	Reference	2.2 ± 0.1
	Polluted	3.2 ± 0.4*
Phagocytosis [No. of zymosan particles (10^7 mg^{-1} protein)]	Reference	44.2 ± 1.3
	Polluted	42.9 ± 1.4

Data are means ± S.E.M., $n = 9$ per site. * indicates a significant difference from the respective reference site (Student *t*-test, $P < 0.05$).

5.3.3 Condition index (CI) and morphometric analysis

Morphometric measurements were recorded from mussels collected from the reference and polluted sites (Fig. 5.5). Shell length values of mussels collected from the polluted site was significantly higher (1.2 fold increase) than mussels from the reference site (Student t-test, $P = 0.001$) (Fig. 5.5). However, the shell weight of reference mussels was higher (1.2 fold increase) than the mussels' shells of polluted site, but not significant (Student t-test, $P > 0.05$) (Fig. 5.5). The total body weight of the mussels from polluted site, as expected, was heavier by approximately 11% than the mussels from the clean site (Fig. 5.5). The flesh weight of mussels from the polluted site was significantly higher (1.3 fold increase) than those from the reference site (Student *t*-test, $P = 0.05$) (Fig. 5.5).Condition index is a measure of the relative proportions of flesh to shell weight. The CI of mussels from the polluted site was significantly less than that of the reference animals (Student *t*-test, $P = 0.03$). Values for CI were 67.0 ± 2.1 and 54.1 ± 4.8 % for animals from the reference and polluted sites, respectively (Fig. 5.5).

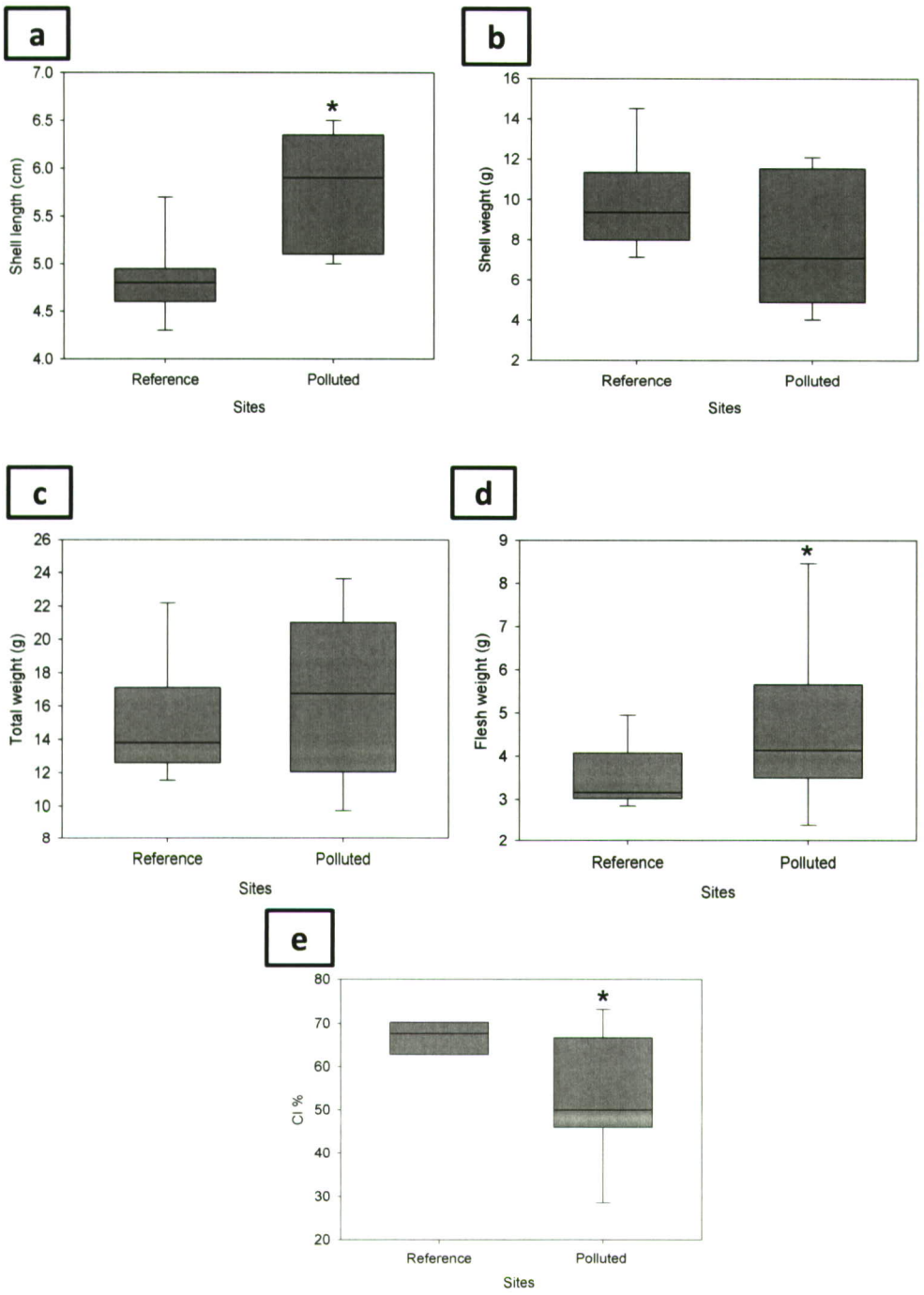

Fig. 5.5 Morphometric measurements illustrating (a) shell length, (b) shell weight, (c) total body weight, (d) flesh weight and (e) condition index of mussels collected from the reference and polluted site, $n = 9$ per site. * indicates a significant difference from the reference site (Student t-test, $P < 0.05$).

5.3.4 Histological alterations

5.3.4.1 Digestive gland

Sections from the reference site did not record any histological abnormality like necrosis or atrophy (Fig. 5.6 and Table 5.4). Total injury and necrosis increased in the animals for the polluted sites (Student *t*-test or Mann-Whitney, $P < 0.001$) and haemocytes infiltration in the connective tissue increased in the animals from the polluted site. The mussels from the polluted site also had a thinner epithelium, values were 64.8 ± 5.0 and 36.2 ± 2.2 in the reference and polluted sites, respectively (Mann-Whitney, $P = 0.001$). All mussels from the polluted site showed alteration in the digestive gland, especially the digestive tubules. The digestive tubules of mussels from the polluted site showed alterations like a decreased tubule diameter (nearly 20% decrease) and foci of inflammation when compared to mussels from the reference site (Student *t*-test, $P = 0.01$, Table 5.4 and Fig. 5.6 a and b).

5.3.4.2 Gills

Almost all the gills from the reference mussels showed normal histology without necrotic or hyperplastic cells. The other group exhibited an increasing trend of gill injury (1.2 fold increase), but this was not statistically significant (Mann-Whitney, $P = 0.2$). Mussels from the polluted site exhibited increased necrotic filaments, especially in the epithelia (Mann-Whitney, $P = 0.02$), erosion of the frontal cilia, but not obvious inflammation (Table 5.4 and Fig. 5.6 c and d).

Table 5.4 Qualitative histological analysis of digestive gland and gills in *M. edulis* collected from the reference and polluted sites

Tissue	Site	Fractional area of tubule (%)	Maximum tubule diameter (μm)	Epithelium diameter (μm)	No. of filaments	Necrosis (%)	Injury (%)	Inflammation
Digestive gland	Reference	82.8 ± 4.4	119.1 ± 5.8	64.8 ± 5.0		4.2 ± 0.2	4.2 ± 0.2	-
	Polluted	63.7 ± 4.4*	95.8 ± 5.7 *	36.2 ± 2.2 *		66.6 ± 0.6 *	74.9 ± 0.6*	+
Gills	Reference				6.0 ± 0.4	13.3 ± 0.5	28.7 ± 0.7	+
	Polluted				6.9 ± 0.3	32.3 ± 2.2*	36.0 ± 0.4	-

Data are means ± S.E.M., $n = 9$ mussels per site. * indicates a significant difference from the reference site (Student t-test or Mann-Whitney, $P < 0.05$).
Note. - indicates absent inflammation, and + rare inflammation.

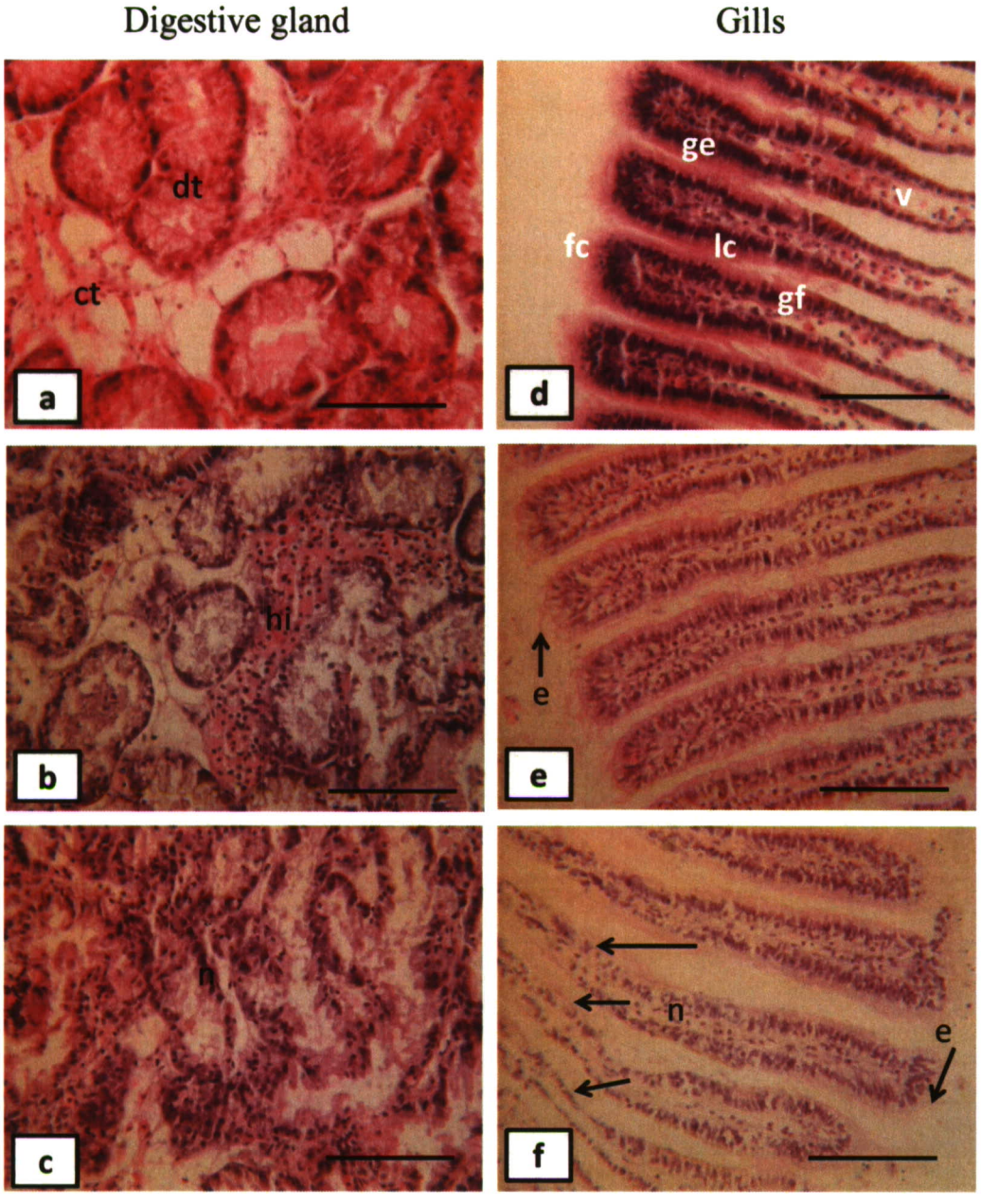

Fig. 5.6 Light micrographs of sections through digestive gland and gills of *M. edulis* showing histological structure of mussels collected from the reference and polluted sites, stained with Haematoxylin and Eosin at 5-10 μm thickness. (a) and (d) digestive gland and gills from the reference site, (b) and (c) digestive gland of polluted site with necrotic tubules and haemocytes infiltration; (e) and (f) gills of polluted site with cilia erosion and necrotic gill filaments with cilia erosion. ct, connective tissue; dt, digestive tubules; n, necrosis; hi, haemocytes infiltration, gf, gill filaments; fc, frontal cilia; lc, lateral cilia; and ge, gill epithelium; v, haemolymph vessel; and e, erosion. Scale bar, 100 μm.

5.3.4.3 Gonads

The male gonads of animals from the reference site showed normal architecture, with well-defined testicular follicles and surrounding connective tissue. There were no signs of necrosis. The sperm tails appear in the middle of the follicle, and the spermatogonia surrounding the peripheral of it. Animals from the polluted sites showed some injuries (Fig. 5.7 Table 5.5). The main injury was mostly as testicular necrosis, which increased significantly in animals from the polluted site compared to the reference site (Student *t*-test, $P = 0.01$). The mussels from the polluted sites appeared to have inflammation and some degeneration of the spermatogonia.

The female gonads of animals from the reference site showed normal architecture, with well defined ovarian follicles and surrounding connective tissue. Necrosis was not recorded. Several oocytes fill the space of the ovarian follicle, and the oogonia surround the peripheral of it. Mussels from the polluted site were characterized by undefined oocytes inside the ovarian follicle plus increased number of granulocytes (inflammation) than the reference one (Table 5.5 and Fig. 5.7 g and h). Necrosis recorded a higher percentage of increase (approximately 64 %) in gonads of mussels from the polluted site over gonads from the reference site. Statistical analysis cannot be applied here because of the limited number of female samples which were collected randomly from the stock aquaria at time zero. The decreased fractional area of ovarian follicles relative to connective tissue was site effect, where fractional areas in mussels from the polluted site (approximately 25 %) were lower compared to those from the reference site (Table 5.5 and Fig. 5.7 e and f).

Table 5.5 Quantitative histological analysis of male and female gonads in *M. edulis* collected from the reference and polluted sites

Gonads	Site	Fractional area of spermatic/egg follicles (%)	Maximum oocyte diameter (μm)	Nuclear material diameter of oocyte (μm)	Necrotic follicles (%)	Inflammation
Male	Reference	55.8 ± 12.1			9.5 ± 0.3	+
	Polluted	55.3 ± 16.9			72.0 ± 0.7 *	+
Female	Reference	70.4 ± 26.5	59.6 ± 8.7	33.4 ± 4.8	16.7 ± 1.0	-
	Polluted	52.9 ± 30.5	63.6 ± 4.8	36.6 ± 4.8	46.7 ± 1.0	++

Data are means ± S.E.M., $n = 9$, except for female gonads, $n = 3$, mussels per site. * indicates a significant difference from the reference site (Student *t*-test or Mann-Whitney, $P < 0.05$). Note. - indicates absent inflammation, + rare inflammation, ++ moderate inflammation.

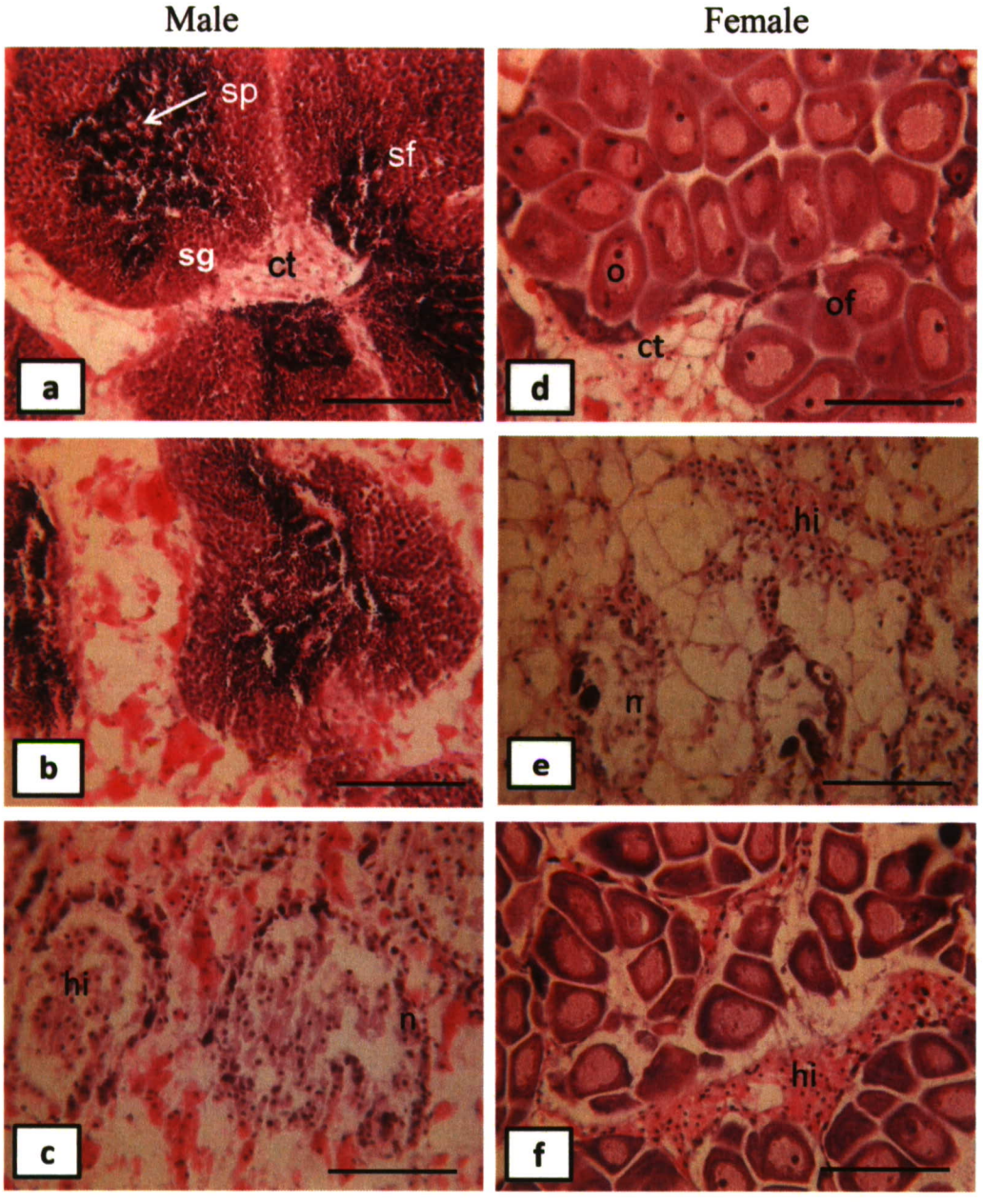

Fig. 5.7 Light micrographs of sections through male and female gonads of *M. edulis* showing histological structure of mussels collected from the reference and polluted sites, stained with Haematoxylin and Eosin at 5-10 µm thickness. (a) and (d) male and female gonads from the reference site. (b) and (c) male gonads of polluted site with inflammatory infiltration and completely necrotic spermatic follicle. (e) and (f) female gonads of polluted site with necrotic and inflammatory egg follicles. ct, connective tissue; n, necrosis; hi, haemocytes infiltration; of, ovarian follicle; o, oocyte; sf, spermatic follicle; sp, sperm flagella; sg, spermatogonies . Scale bar, 100 µm.

5.3 Discussion

This study evaluated pre-exposure history by measuring metal accumulation, cellular immunity, mussels' physiology (CI), and histopathology in the same exploration comparing the clean site to the polluted site. The study recorded significant changes in immunological functions of haemocytes (neutral red uptake), tissue pathologies, and adverse effects on mussels CI and morphometrics as a result of pollution. In this study we evaluated pre-exposure history effect on cellular immune function, mussels physiology and organ pathology in one investigation *in vivo*.

Pollutant exposure was confirmed by an increase of trace metal levels in the mussels' tissues (Table 5.2). In addition, electrolytes of haemolymph were disturbed as an effect of the pollution in the mussels from the polluted site (Table 5.2). When comparing our findings with Turner et al. (2009) on Cu and Zn in gills of *M. edulis* collected from Plymouth Sound, we found out that our levels of the same metals, organ were much higher. Our metal analysis data recorded an increase in non-essential and a decrease or disturbance in the essential metals as an effect of site contamination. Similar findings were recorded by Roesijadi et al. (1984) field study on *M. edulis,* who found an increase in metals like Hg, Zn, Cu, Cd and Ag in the contaminated site compared with the pristine site in Washington, USA.

Measurements of metals in sediment (Table 5.1)/tissues (Table 5.2) showed that the polluted site has higher concentrations of Cu, Fe, Zn and Pb in comparison to the reference site. This is in consistence with Roesijadi et al. (1984), who compared two mussels' tissue and seawater metal concentration between a clean region and an impacted one (Sequim and Tacoma, Washington, USA) and recorded a significant increase of metals in the polluted site. Vlahogianni et al. (2007) recorded 3-4 fold increased trend of Fe, Cu and Pb accumulation in *M. galloprovincialis* tissues from

polluted sites when compared to the control site (Saronikos Gulf, Greece) depending on the season. In a study on six sites in the UK by Dyrynda et al. (1998), soft tissues from mussels collected from Dorset (Poole and Holes Bays) recorded high body burdens of Cu, Pb, Zn and Cd than other metals. However, the site in Dorset has similar shipping activity like the site in Plymouth; Cd and Pb levels were higher than our data. Zn and Cu concentration in the site of Dorset were similar to our values.

In our survey, the contamination affected and reduced Ca levels in the haemolymph, digestive gland and gills of mussels from the polluted site (Table 5.2). Schoenmakers et al. (1992) reported cadmium (IC50 of 8.2 pM) affects Ca^{2+} ATPase in tilapia intestine and as a result inhibiting the calcium pump. Verbost et al. (1988) reported the same effect of Cd in fish gills where Cd effect was competitive with Ca^{2+} binding sites and increased free Ca^{2+} levels. In the current study, Ca^{2+} levels increased and necrosis was a general pathology in gonads tissue of the polluted site over the reference site animals and elevation of Ca^{2+} in cells leads to necrosis as described by Orrenius et al. (1989).

Neutral red uptake in haemocytes from the polluted site mussels showed a significant increase, however, inhibition in phagocytic activity was recorded in the polluted site mussels compared with the reference site (Table 5.3). Galloway et al. (2002) recorded a disturbance in biological response including neutral red retention time % according to site pollution levels. As Luengen et al. (2004) mentioned that contaminated animals suffer from food shortage in their environment and thus decrease their haemocytes phagocytosis ability. Lysosomal alterations can be attributed to an increase/decrease of lysosomal membrane permeability to specific substances as a result of contaminant exposure (Hawkins, 1980) or an increase in the volume of lysosomal vacuolar system (Pickwell and Steinert, 1984). An increase in immune response is one

of the ways of detoxification to eliminate contaminants (Ruddell and Rains, 1975). So, our mussels experienced contamination stress like what happened in neutral red uptake. Impairment in the immune cellular responses of *M. edulis* under the effect of single or mixture of xinobiotics exposures (*in vitro/in vivo*) reported by (Grundy et al., 1996a and b). They recorded inhibition in phagocytic ability and disturbance in lysosomal membrane permeability of the haemocytes. The adverse effect can be reached when the organism fails to respond positively to toxicant exposure. The idea of toxicity of metals has been explained by Brown and Parsons, (1978) as spillover of metals when metal-binding protein capacity is exceeded after exposure.

Our data indicated a significant decrease in the polluted mussels` CI when compared to clean mussels (Fig. 5.5). Similar findings were recorded by Roesijadi et al. (1984); Roméo et al. (2005) and Kljaković-Gaspić et al. (2006), who found significant effects of pollution on CI of *M. edulis* and *M. galloprovincialis*, respectively. By contrast, Galloway et al. (2002) did not observe any change between mussels` CI among sites. One reason for lower CI in the polluted site compared with the clean site may be the tissue lesions of the examined organs or disturbance in the shell /flesh weight as a result of metals accumulation.

A significant correlation has been recorded between shell length and some metal concentrated in tissues of mussels and not with others (Figs. 5.2, 5.3 and 5.4). Our finding is in accordance with the previous report of Brown and Luoma (1995). They found significant correlations between shell size and some element concentrations in the euryhaline bivalve *Potamocorbula amurensis* collected from clean and contaminated sites. However, they attributed these correlations to the wide size range of sampling and collecting one size animals reduce these correlations and mislead its interpretations. In more recent study, De Wolf et al. (2000) found no correlation between shell size and

metal pollution in the periwinkle *Littorina littorea*. They explained the reason of this as pollution/salinity threshold and shell size.

Tissue examination revealed abnormal and pathological features in all examined organs from the contaminated site (Figs. 5.6, 5.7 and Tables 5.4, 5.5). Fe and Pb were confirmed to be significantly high in all histologically examined tissue (Table 5.2). Furthermore, a decrease in the Ca^{+2} content of examined tissues confirmed disturbance in the Ca^{+2} channels of mussels from the polluted site (Table 5.2). Inflammation and necrosis were the most characteristic symptoms of the digestive gland of mussels from the polluted site. In an early study by Lowe and Moore (1978), *Mytilus edulis* mussels collected from the River Lynher at Plymouth, England. They found the connective tissue of digestive gland is invaded or completely replaced by enlarged, atypical, mitotically active, basophilic, hemocyte-like cells. They explained these lesions as an early stage of a disease related to the presence of carcinogenic aromatic hydrocarbons detected in the mussels' bed. Contaminated field samples of *M. edulis* digestive tubules epithelium were confirmed to be the location of inflammation and necrosis (Auffet, 1988). Da Ros et al. (1995) also found significant thinning in the digestive tubules of *Mytilus.* sp. in the treated groups with Cd. Similar findings reported by Auffret (1988) in the digestive gland of *M. edulis* from a contaminated Norwegian fjord as interstitial granulosytomas (haemocytes aggregation). He added that granulosytomas are a symptom of long-term exposure to a contaminant. Granulocytoma was found to be higher in mussels' tissue of the impacted area with pollution (Svärdh and Johannesson, 2002). Cd also can cause a significant increase in Ca^{2+} concentrations in the ventricular fluid in the freshwater bivalve *Anodonta cygnea* (Faubel et al., 2008), and loss of Ca^{2+} homeostasis is a key step in initiating cellular necrosis. In an earlier study, Neff et al. (1987) detected neoplasm and granulocytomas in bivalve digestive gland and male gonads after oil spill experiment. Pollution evoked necrosis and cilia loss in mussel gill

filaments. Inflammation, oedema and loss of lateral cilia of gills are common features associated with heavy metal exposure (Sunila, 1988). David et al. (2008b) demonstrated pathological symptoms in gill filaments of *Mytella falcata* collected from a polluted environment in the Santos estuary. Their findings confirm ours; inflammation and epithelial detachment was the major pathology of pollution in their study. It is suggested that all histological abnormalities of the gill filament epithelium may affect the cell membrane permeability, and consequently the function of the gills. Necrotic follicles, degenerated gametocytes and inflammatory reactions in gonads of mussels from the polluted sites may affect the mussels' maturity or reproduction process. Myint and Tyler (1982) confirmed metal exposure plus external factors such as temperature and nutrition can affect successful reproduction of *M. edulis* from Swansea Bay, South Wales (UK).

In the present study, many parameters have been measured to assess the health status and to confirm the adverse effects of pollution on mussels. Some of these parameters were evidently more sensitive than others. Neutral red retention as a reliable tool of cells health, and histological examination as a snapshot picture of what was/is happening to the organisms` tissues proved an excellent connection to pollution effects.

5.6 Conclusion

Mytilus edulis mussels were collected from the reference site, Port Quin and the polluted site, Mount Batten. A site effect was observed in this study, where the polluted site showed poorer biological measurements than the clean site. *M. edulis* tissues from the polluted site have higher Fe and Pb and to a lesser extent with Cd, Cu, Zn and Ca in the gills, digestive gland, gonads, and haemolymph compared to the clean site. This study demonstrated that tissue abnormalities were related to chemical contamination. Haemocyte functions like neutral red uptake and phagocytosis were also affected by pollution and recorded stimulation/inhibition responses. Haemolymph and tissue electrolytes of mussels from the polluted site showed significant disturbance compared with the reference site. This study suggested that measurements of trace metals in seawater and sediments of the investigated sites are important. Our study suggested that quantitative histology, rapid immunoassays and tissue burdens were good and integrated tool in the assessment of pollution and its adverse effects *in vivo*.

Chapter 6

Effects of pre-exposure history and cadmium exposure on immunological responses, organ histology and gene expression in the marine mussel, *M. edulis*

Chapter 6

Effects of pre-exposure history and cadmium exposure on immunological responses, organ histology and gene expression in the marine mussel, *M. edulis*

Abstract

The effect of exposure history on the immunological response to a new metal exposure is poorly understood. The marine mussels, *Mytilus edulis*, were collected from reference and polluted sites in South-West England and groups of mussels from each site were exposed to 20 μg l^{-1} $CdCl_2$ for 0, 1, 4, and 8 days compared to unexposed controls. End points included tissue metals/electrolytes, haemolymph chemistry, haemocyte counts, neutral red uptake (NR), phagocytosis assay and histology. Exposure to Cd in the laboratory caused Cd accumulation mainly in the digestive gland, and there was a site-effect on trace metals. NRR, the phagocytosis assay, condition index (CI) and haemolymph Na or K levels showed some changes between sites and Cd-treatment; but with no clear trends. Gills, digestive gland and haemolymph showed lower Ca levels in animals from the polluted sites with Cd-exposure, compared to controls (approx. 30, 28 and 5 % lower than controls, Kruskal Wallis, $P < 0.05$). Some correlations were found between Ca concentration in the digestive gland and Cu ($R^2 = 0.4$, $P = 0.04$), but not other metals. Histological examinations showed tissue necrosis and inflammation in animals from the polluted sites, but Cd exposure (unexpectedly) improved some of these pathologies. We conclude that metal pollution modulates immunity in *M. edulis*, but pre-exposure history has limited effects on these responses.

Chapter 6

6.1 Introduction

Chronic exposure to low level pollution will inevitably cause deterioration in organism health and may lead to toxicity (Rasmussen et al., 1983; Usheva et al., 2006). The toxic effects of chronic pollution have been demonstrated at the biochemical, physiological and behavioural level in marine organisms (Sant'Ana et al., 2005; Berntssen et al., 2003). For example, in the marine bivalve, *Macoma balthica*, burrowing and avoidance were impaired after chronic exposure to heavy metal pollutants (McGeer, 1979). Chronic Cd exposure for 1 year in *M. edulis* causes dilatation of gills veins and fusion of different parts of the filaments (Sunila, 1986).

One way of demonstrating the adverse effects of a polluted ecosystem is to collect animals from a polluted site and compare their health with animals from a nearby clean reference site (Chapter 5, Handy et al., 2003). However, it is also possible to move animals from polluted sites to clean ones, or indeed, transplant animals from clean to polluted sites for observation (Ruddell and Rains, 1975; Pipe et al., 1995b; David et al., 2008b). Studies have been done on depuration ability of the organisms after being transferred from a polluted environment to clean conditions (Livingstone, 1991). In some cases, the biological functions of the organisms can improved, but full recovery is often unlikely because some injuries may not repair even if the pollutant has been excreted from the body (Depledge and Fossi, 1994). This raises the possibility that animals from polluted sites will be less able to respond to a new exposure to pollutants.

Mussels from contaminated areas may be suffering from a shortage of food (food stress) and this consequently affects their biological responses like phagocytosis (Luengen et al., 2004). Ingersoll and Winner (1982) tested chronic exposure to Cd/Cu on the freshwater *Daphnia pulex* as pulses for 70 days and found Cu exposure caused a significant reduction in growth, survival and reproduction. Pascoe and Shazili (1986)

ran brief Cd exposures (32 min) followed by restoration to clean water or continuous exposures (1900 min) on the rainbow trout. Pascoe and Shazili (1986) indicated that brief exposure followed by restoration to clean water caused post-exposure mortalities as result of low concentrations of Cd. In addition, a marked reduction in Cd concentration of fish tissue was recorded after 24h in restored fishes compared to Cd concentrations after the exposure and % mortality increased with time Cd exposure. The chronic sublethal effect of intermittent exposure was studied by Sayer (1991) in embryonic and larval stages of *Salmo trutta* as episodic exposure to acid, aluminium and copper in soft waters. The author found poor growth rates as an effect of acid exposure. A different study has been done on macro-invertebrates by Merrett et al. (1991) who exposed macro-invertebrates to chronic exposure of 29 days of different acidity and stimulated acid episodes with aluminium sulphate. They found that the mortality increased with aluminium exposure and decreased in pH, but the effect was different between species. However, in the episode regime mortality depends on increased episode duration. Handy (1994) reviewed intermittent exposure to aquatic pollutants in fish and invertebrates, its toxicity and sublethal responses.

M. edulis inhabits the sediment surface area of Plymouth Sound which is contaminated with metals like Cu and Zn (see Chapter 5). The aim of this study is to determine if the effect of exposure history (i.e., animals from reference and polluted sites) altered the response to subsequent Cd exposure in the laboratory. The endpoints include cellular immunity (cell viability, phagocytosis), physiological status (condition index), histopathology (organ histology), and gene expression regulation in the digestive gland (RT-PCR, Chapter 7) as well as metal concentrations (e.g., Cd accumulation).

6.2 Materials and methods

6.2.1 Experimental design

This experiment was aimed to study the effect of Cd as $CdCl_2$ exposure on the immune system, organ pathology and physiology of mussels collected from clean and naturally polluted sites. A Cd concentration of 20 $\mu g\ l^{-1}$ Cd as $CdCl_2$ was used in this experiment. The measured end points were condition index, neutral red assay, phagocytosis, tissue and haemolymph Na, K, Ca, Pb, Zn, Fe, Cu, , *mt10* gene expression (mRNA, Chapter 7) and histological abnormalities of gills, digestive gland and gonads at 24 h, 4, and 8 days. Mussels were divided into four groups (Fig. 6.1), reference control mussels; reference + Cd mussels; polluted control mussels and polluted + Cd mussels. A total of 108 mussels were used (12 animals/tank, in triplicate = 36 animals/treatment).

Routine water quality, haemolymph chemistry, haemocyte counts, tissue collection, metal analysis, immunological assays like neutral red and phagocytosis, as well as histology were measured as detailed in Chapter 2. Condition index was determined as shown in Chapter 5.

Seawater quality was confirmed by measured values during the experiments as follows (data are mean ± S.E.M., n = 48): % dissolved oxygen (98.1 ± 0.3), pH (7.7 ± 0.02), total ammonia (0.03 ± 0.01 mg l^{-1}), salinity (30.8 ± 0.01 parts per thousand), temperature (14.8 ± 0.1 °C), and photoperiod 12 light: 12 dark. Cd exposure was confirmed by measuring the Cd in treated tanks and values were 20.2 ± 1.0 and 20.0 ± 3.0 for reference + 20 $\mu g\ l^{-1}$ $CdCl_2$ and polluted + 20 $\mu g\ l^{-1}$ $CdCl_2$ tanks, respectively. Background levels in the tanks not treated with Cd were < 4 ± 0.5$\mu g\ l^{-1}$ Cd.

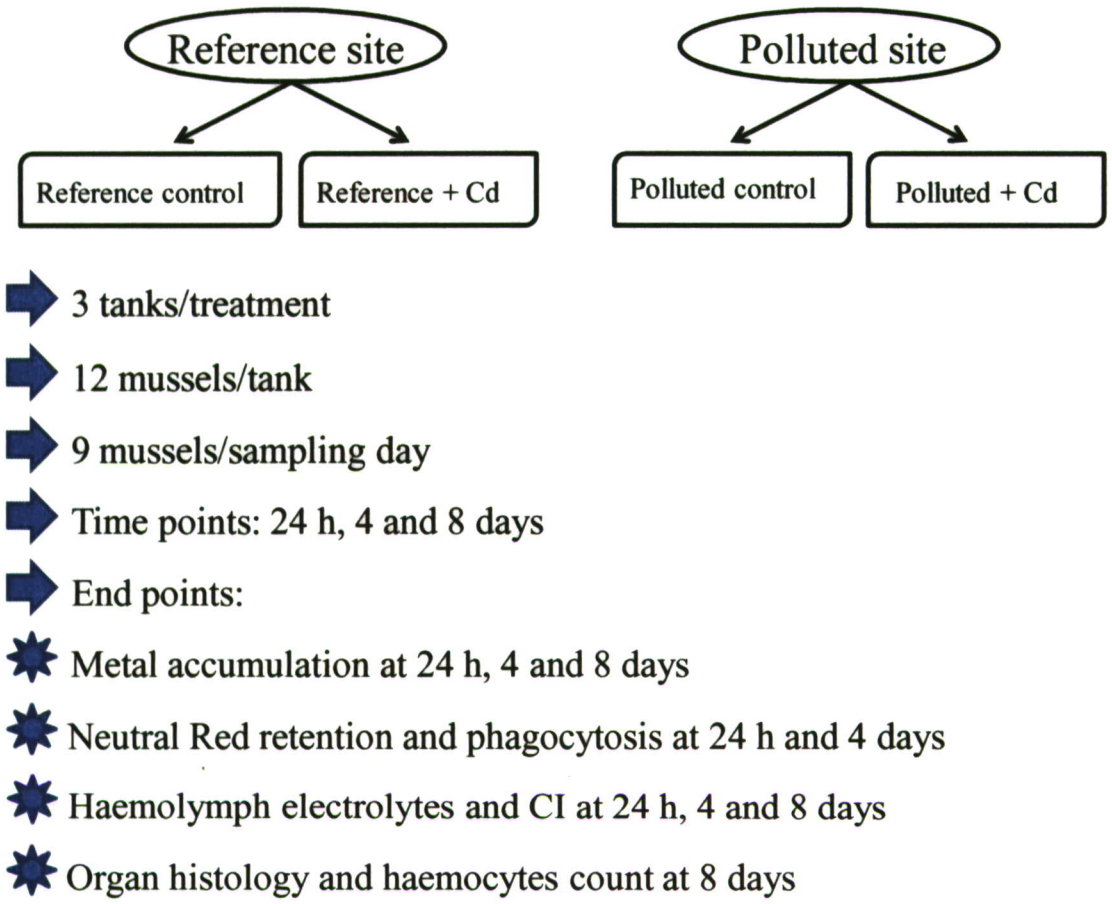

Fig. 6.1 Diagram showing the experimental design. Animals from each field site were split into two groups (controls, and Cd-exposed). Sampling times, numbers of mussels and end points are indicated.

6.2.2 Statistical analysis

All data were analysed using Statgraphics 5.1 Plus software, with a rejection level of $P = 0.05$. No tank effects were observed within triplicates, so data were pooled by treatment for statistical analysis. Data were analysed using two-way ANOVA with the factors treatment, site, and treatment × site and factors were included in the model if they were significant ($P < 0.05$). Simple effects in some data were evaluated by one-way ANOVA within time, or for treatment effects. In all analyses, homogeneity of variance was assessed (Barlett's test) and the Least Squares Difference (LSD) test was used to locate specific treatment and time effects within ANOVAs. Where ANOVA could not be applied, a non-parametric ranking test was used (Kruskal Wallis test). All data are expressed as means ± S.E.M. for $n = 9$ mussels per treatment/site, unless otherwise stated. Correlations between trace metals were determined using Pearson's correlation coefficient. Correlation plotting and fitting the best curves to the data were carried out using SigmaPlot 11.0 software.

6.3 Results

6.3.1 Metal accumulation during cadmium exposure

Groups of mussels collected from reference and polluted sites were exposed to 20 μg l^{-1} Cd. There were no mortalities in the experiment. There were statistically significant differences (Kruskal Wallis, $P = 0.005$) in the cadmium content of tissues (digestive gland > gill > gonads) (Fig. 6.2). Cadmium accumulation found to be significantly higher in the gonads of mussels from the polluted control site (Kruskal Wallis, $P = 0.03$), than the other tissues. Cadmium exposure caused large (3, 10 and 6 fold) increases in Cd content of polluted + Cd mussels tissues compared to the control polluted animals without Cd exposure at the beginning 24 h of the experiment (Fig. 6.2). The pollution + Cd effect was statistically significant at all time points, compared

to the reference control or the polluted control (Kruskal Wallis, $P < 0.01$). A time-dependence of Cd accumulation was found to be statistically significant in both the reference + Cd and polluted + Cd groups (Kruskal Wallis, $P < 0.001$). A significant interaction in site × treatment on day 8 was recorded (two-way ANOVA, $P = 0.01$), indicating that the field collection site did alter the Cd accumulation response in the laboratory by the end of the experiment in digestive gland. The haemolymph showed a significant change (with no consistence trend) in Cd content as an effect of treatment with Cd (ANOVA, $P < 0.001$) compared to all other groups only at 24h (Fig. 6.2d). Also, time-dependent effects were recorded in the reference + Cd, mussels (ANOVAs, $P < 0.01$). Two-way ANOVA on day 8 revealed no significant interaction in site × treatment ($P = 0.06$) for haemolymph Cd.

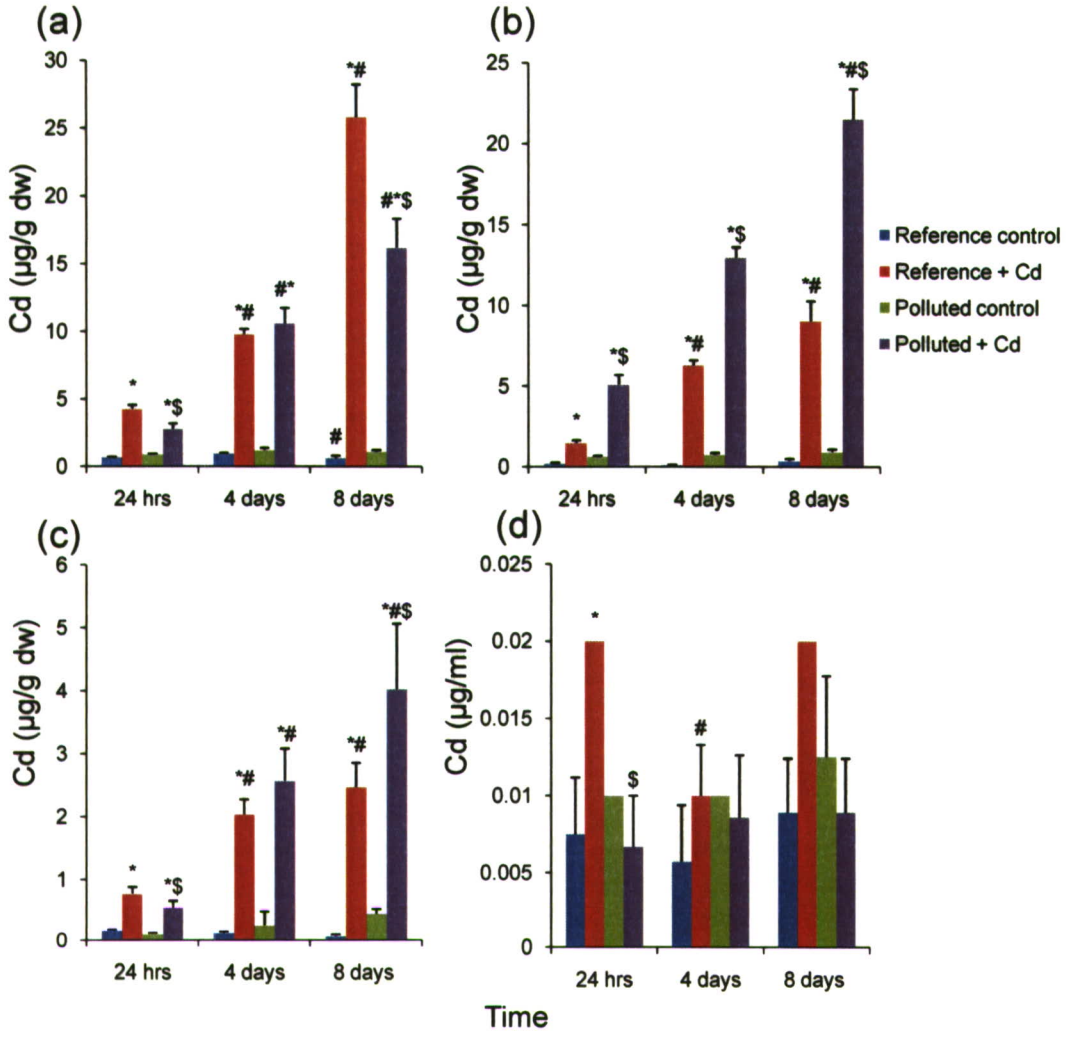

Fig. 6.2 Cd concentrations in digestive gland (a), gills (b), gonads (c) and haemolymph (d) in *M. edulis* reference control, reference + Cd, polluted control and polluted + Cd. Data are means ± S.E.M., n = 9 per treatment at each time point. * indicates a significant difference from the respective control within field site. $ indicates a significant difference in Cd effect between sites. # indicates a significant difference in time within treatment, $P \leq 0.05$, ANOVA or Kruskal Wallis.

There were differences in the calcium content of tissues (gill > digestive gland > gonads) (Fig. 6.3). Calcium content showed site effect even after Cd treatment, and were found to be significantly lower in the gills and digestive gland of polluted control and polluted + Cd mussels compared with the reference control (Kruskal Wallis, P = 0.01) at 24 h and on day 4. However, gonadal tissue Ca content did not show a statistically significant change by site or treatment. Time-dependent effects were only found to be significant in the gills of polluted control animals (Kruskal Wallis, P = 0.001). Two-way ANOVA analysis on day 8 recorded no combined effect of site × treatment (P = 0.8) for gills levels of Ca. Also, Cd treatment has a significant effect on Ca content in the reference + Cd and polluted + Cd mussels' haemolymph when compared to reference and polluted controls mussels (ANOVA, $P < 0.01$) (Fig. 6.3d). Also, time-dependent effects within treatment were recorded in polluted + Cd mussels group, but no site × treatment effects were found (ANOVA or Kruskal Wallis, $P <$ 0.001, two-way ANOVA, $P = 0.5$).

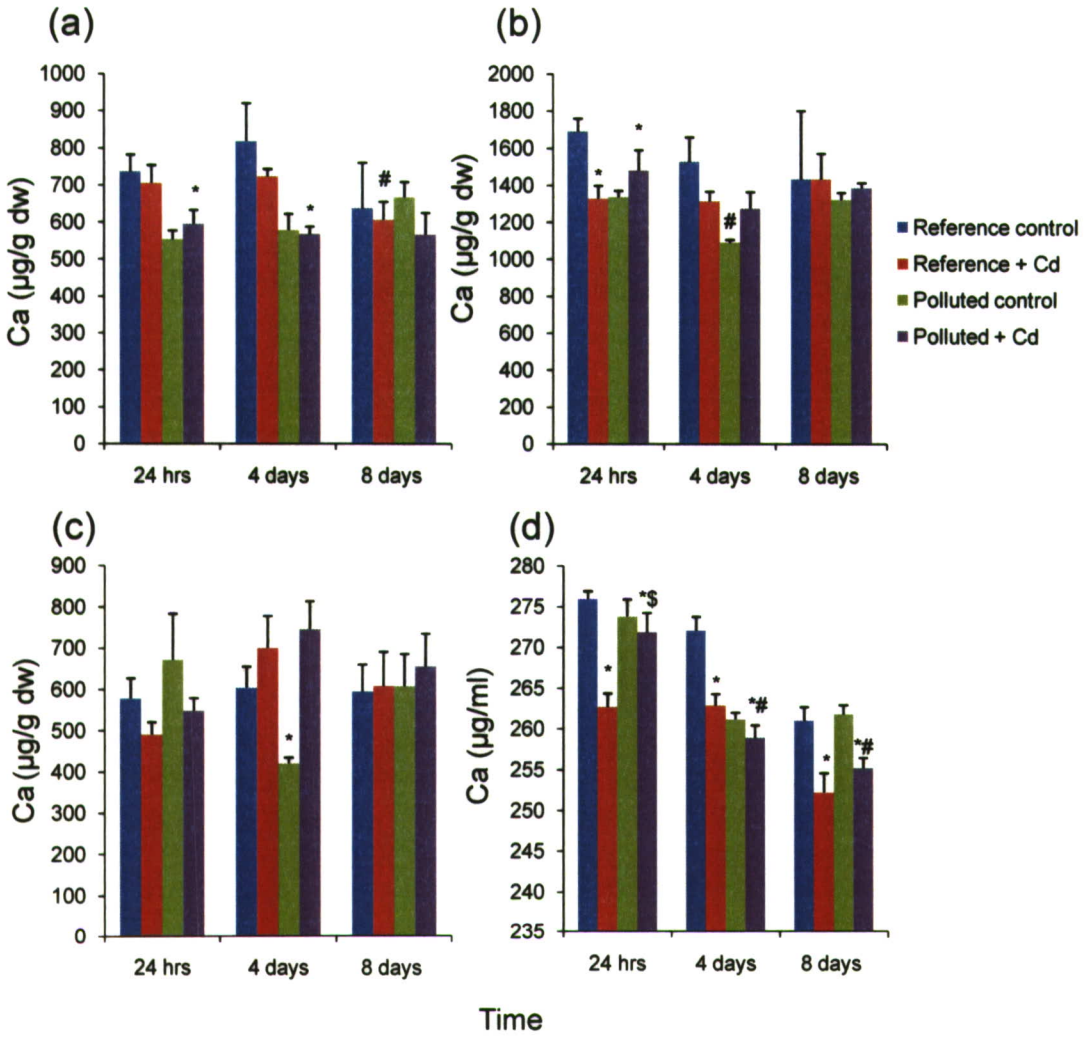

Fig. 6.3 Ca concentrations in digestive gland (a), gills (b), gonads (c) and haemolymph (d) in *M. edulis* reference control, reference + Cd, polluted control and polluted + Cd. Data are means ± S.E.M., n = 9 per treatment at each time point. * indicates a significant difference from the respective control within field site. $ indicates a significant difference in Cd effect between sites. # indicates a significant difference in time within treatment, $P \leq 0.05$, ANOVA or Kruskal Wallis.

A significant combined effect between site × treatment on day 8 was found (two-way ANOVA, $P = 0.01$) for digestive gland Zn concentrations. There were significant differences (Kruskal Wallis, $P = 0.00001$) in the Zn content of tissues (digestive gland > gill > gonads) (Fig. 6.4a). Zn content found to be significantly higher in the digestive gland and gonads in the reference + Cd than the reference control and polluted + Cd (Kruskal Wallis, $P < 0.02$) on 8 days. However, in contrast, the gills tissue content of Zn was not statistically significant but elevated in the polluted + Cd compared with all other groups. Haemolymph Zn levels showed no significant difference between reference control and reference + Cd groups (Kruskal Wallis, $P = 0.2$) at 8 days (Fig. 6.4b). However, an obvious treatment effect can be seen in the reference + Cd groups with Cd as not significant decrease in concentration when compared to its respective control. Interaction in site × treatment data recorded no significant effect at 8 days (two-way ANOVA, $P = 0.8$) for haemolymph.

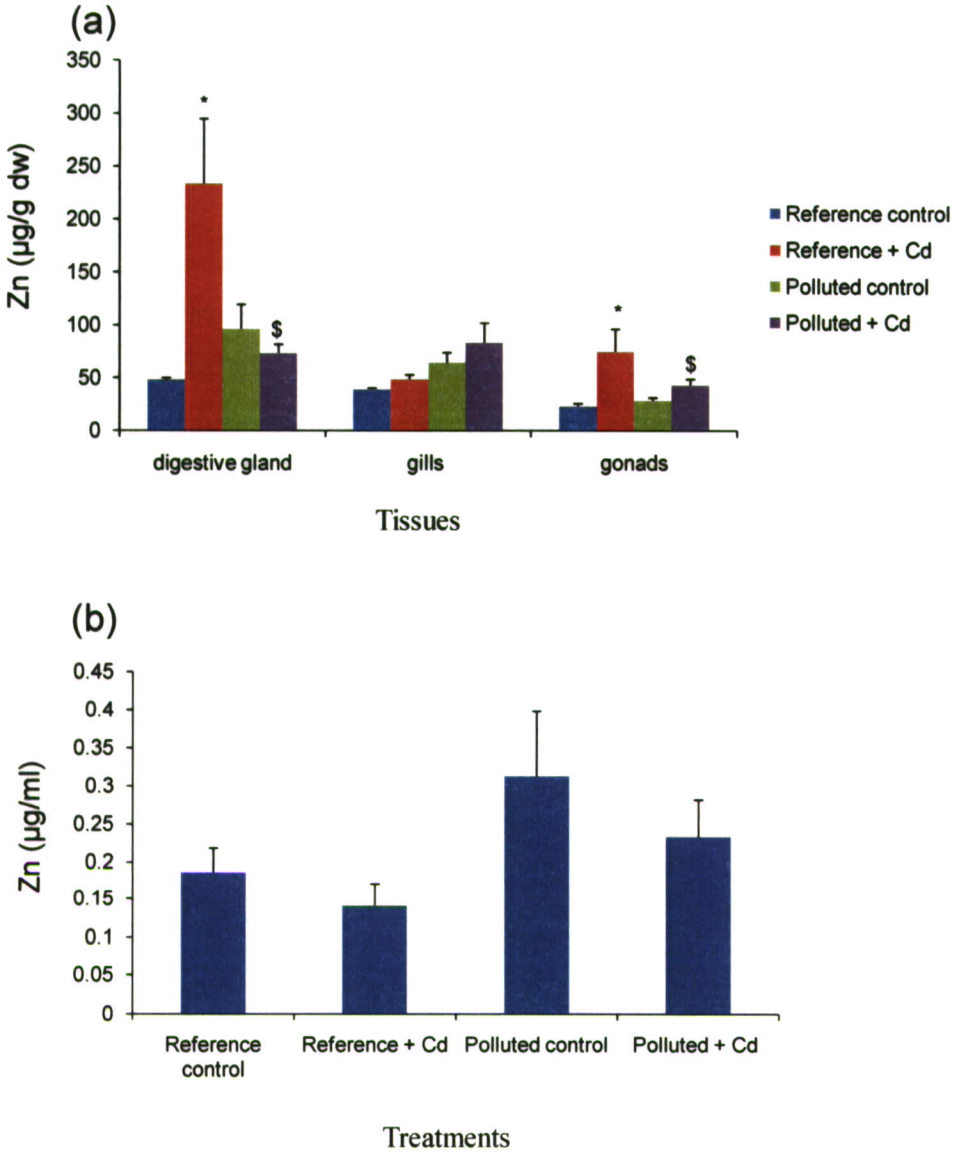

Fig. 6.4 Zn concentrations in digestive gland, gills and gonads (a) and haemolymph (b) in *M. edulis* reference control, reference + Cd, polluted control and polluted + Cd at 8 days. Data are means ± S.E.M., n = 9. $ indicates a significant difference in Cd effect between sites. * indicates significant difference from the respective control within field site, $P \leq 0.05$, ANOVA or Kruskal Wallis. No statistically significant effects were observed in panel b.

At the end of the experiment, Cu content was found to be the highest in the gills > digestive gland > gonads (Fig. 6.5a). The digestive gland and gonads showed no significant treatment or site effects on Cu accumulation (Kruskal Wallis, $P > 0.05$). On the other hand, the gills showed a significant decrease in Cu content according to site effect, and site + treatment effects compared to the reference + Cd mussels (Kruskal Wallis, $P < 0.001$) at 8 days with no site × treatment trend detected using two-way ANOVA on day 8 ($P = 0.2$) for digestive gland Cu content. The haemolymph content of Cu was significantly higher in polluted + Cd mussels (approx. 80 %, Kruskal Wallis, $P = 0.006$) compared to the polluted control and reference + Cd mussels, respectively on day 8 (Fig. 6.5b). From Fig. 6.5a, Cd treatment decreased Cu levels in reference + Cd and polluted + Cd mussels' haemolyph when compared to reference control and polluted control mussels, respectively. Two-way ANOVA analysis at the end of the experiment found no significant interaction in site × treatment ($P = 0.6$) for haemolymph.

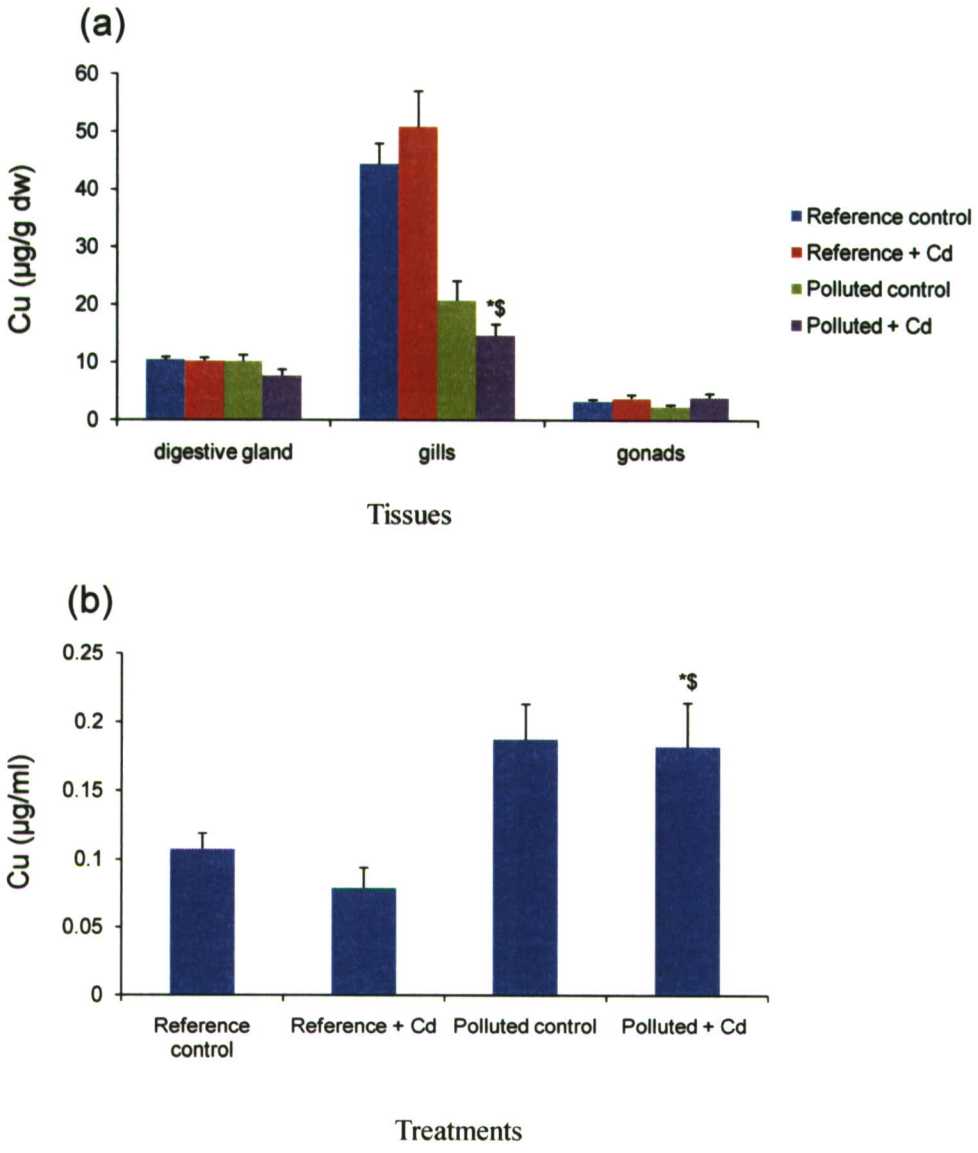

Fig. 6.5 Cu concentrations in digestive gland, gills and gonads (a) and haemolymph (b) in *M. edulis* reference control, reference + Cd, polluted control and polluted + Cd at 8 days. In panel (a), (b), (c) and (d) data are means ± S.E.M., n = 9 per treatment at each time point. $ indicates a significant difference in Cd effect between sites. * indicates a significant difference from the respective control within field site, $P \leq 0.05$, ANOVA or Kruskal Wallis.

Fe content was measured at the end of the experiment at 8 days. Fe levels in tissues were as the following order, gills > digestive gland > gonads (Fig. 6.6a). Fe tissue burden was affected by site not by treatment. All examined tissues (digestive gland, gills and gonads) from polluted control and polluted + Cd mussels showed significant increase of Fe concentration compared to the reference control and reference + Cd mussels alone (Kruskal Wallis or ANOVA, $P < 0.01$). Also, on day 8 site × treatment combined effect was found significant (two-way ANOVA, $P = 0.05$) for Fe levels in gills. No significant difference in Fe content of haemolymph was found between any of the tested groups (ANOVA, $P = 0.1$) and no significant interaction effect recorded in site × treatment (two-way ANOVA, $P = 0.8$) for haemolymph on day 8. However, the most decreased groups were polluted control and polluted + Cd mussels (Fig. 6.6b).

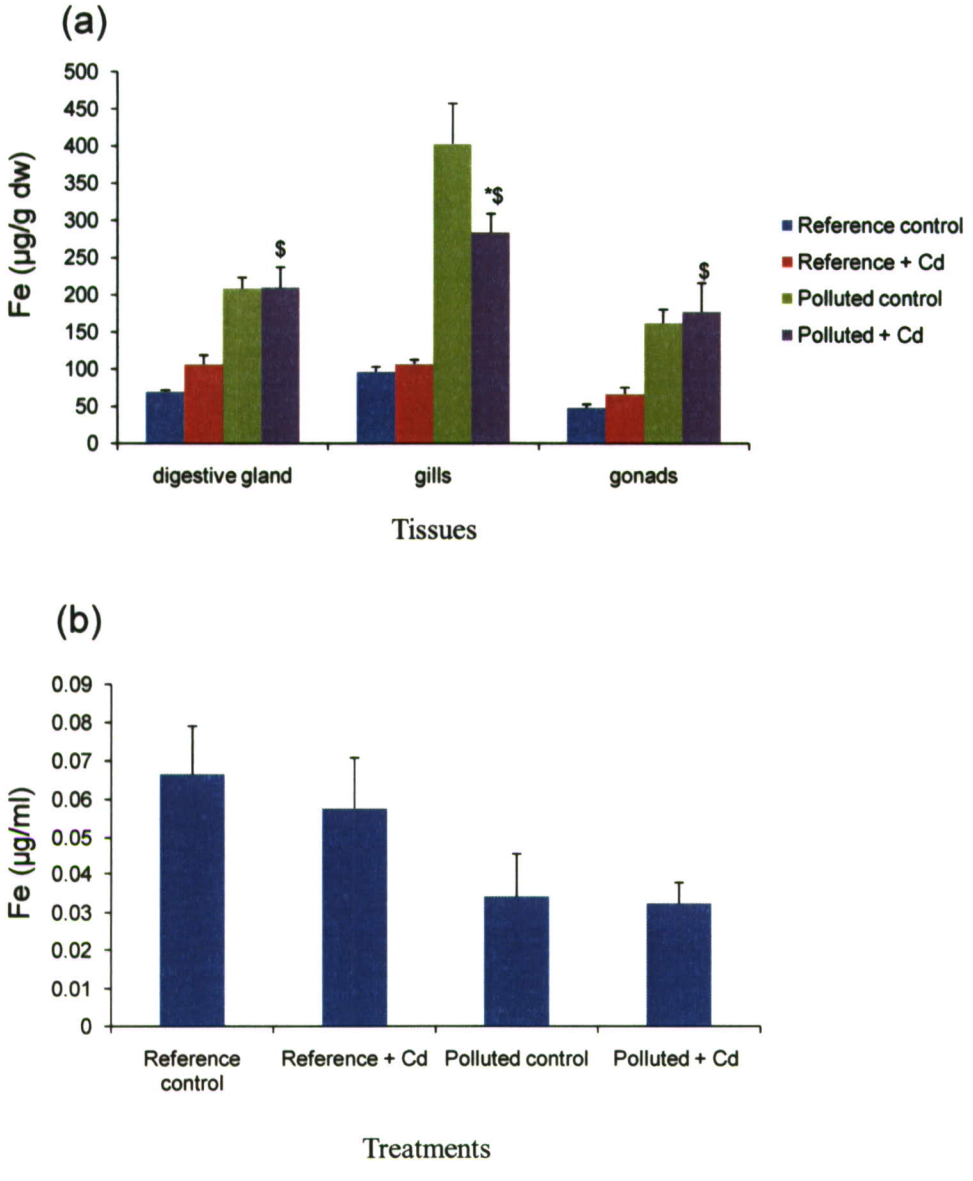

Fig. 6.6 Fe concentrations in digestive gland, gills and gonads (a) and haemolymph (b) in *M. edulis* reference control, reference + Cd, polluted control and polluted + Cd at 8 days. Data are means ± S.E.M., n = 9. * indicates a significant difference from the respective control within field site. $ indicates a significant difference in Cd effect between sites, $P \leq 0.05$, ANOVA or Kruskal Wallis.

Pb tissue content was measured at the end of the experiment at 8 days. Pb levels in tissues was as the following order, gills > digestive gland > gonads (Fig.6.7a). Pb tissue burden recorded higher levels by the effect of site not by treatment, especially in the gills of polluted control and polluted + Cd mussels when compared to reference control and reference + Cd mussels. However, Pb content of the gonads increased in the polluted + Cd mussels under the effect of Cd treatment compared with reference control and reference + Cd (Kruskal Wallis, $P < 0.05$). Two-way ANOVA analysis of Pb content in gills on day 8 recorded no significant combination in site × treatment ($P = 0.1$). No significant difference was found in Pb levels in haemolymph, but the haemolymph from polluted control mussels recorded the highest levels compared to all other groups but not significantly (ANOVA, $P = 0.5$) and no combined effect of site × treatment (two-way ANOVA, $P = 0.6$) (Fig.6.7b) of haemolymph. As shown in Fig. (6.7b), there were decreased Pb concentrations in reference + Cd and polluted + Cd compared to reference control and polluted control mussels.

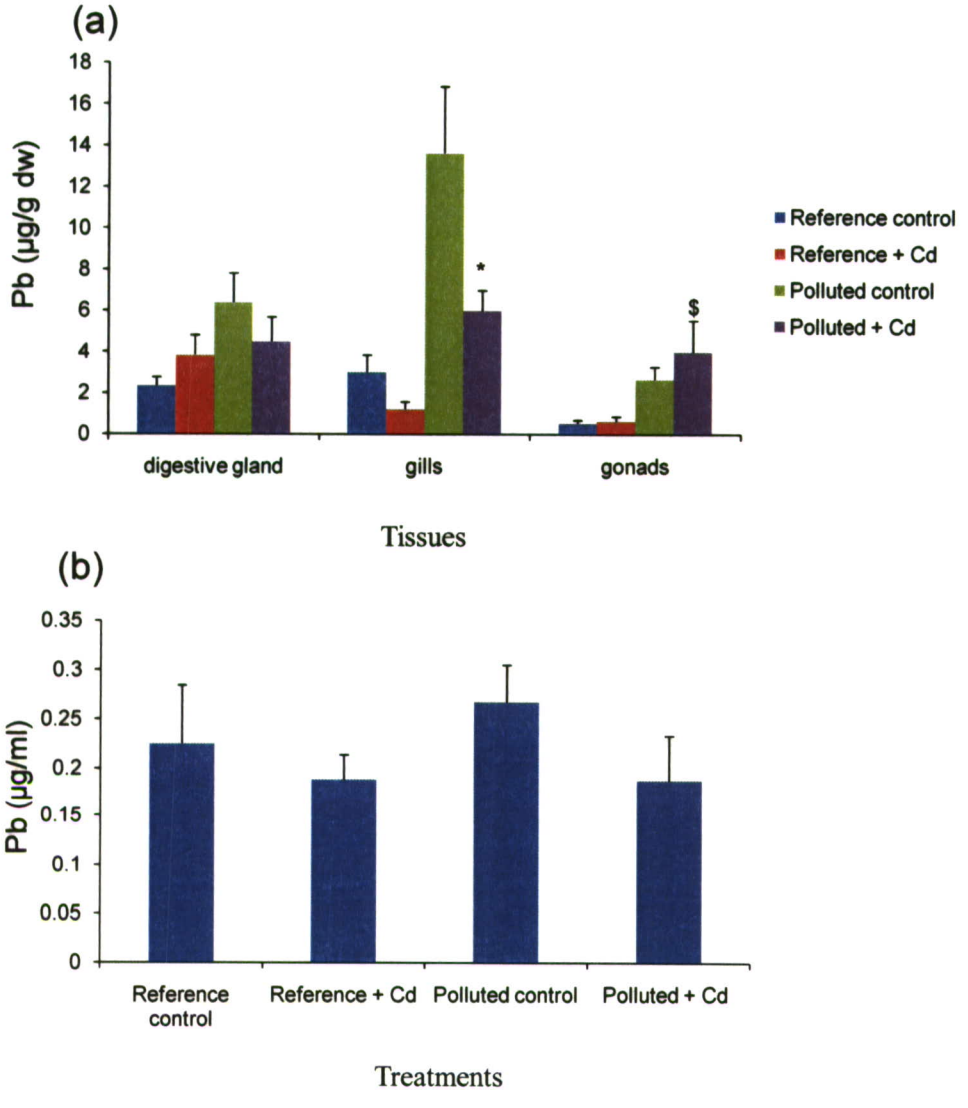

Fig. 6.7 Pb concentrations in digestive gland, gills and gonads (a) and haemolymph (b) in *M. edulis* reference control, reference + Cd, polluted control and polluted + Cd at 8 days. Data are means ± S.E.M., n = 9. $ indicates a significant difference in Cd effect between sites, $P \leq 0.05$, ANOVA or Kruskal Wallis.

Statistical values demonstrated relatively strong correlations between the essential metals like Cu and Ca concentrations in the digestive gland (Pearson's, P = 0.04). However, a weak correlation was found between Zn concentration in the digestive gland and Fe concentration in the gills and Ca concentrations (Pearson's, $P >$ 0.05) (Fig. 6.8). Reference control and reference + Cd mussels were clustered in a separate group to the polluted control and polluted + Cd mussels' as shown in Fig. (6.8c). Furthermore, there was a weak correlation between the non-essential metals like Cd concentration in the digestive gland, Pb concentration in the gills and Ca concentrations (Pearson's, $P > 0.05$) (Fig. 6.8). As shown in Fig. (6.8), the reference control and polluted control mussels were grouped together separately from reference + Cd and polluted + Cd mussels.

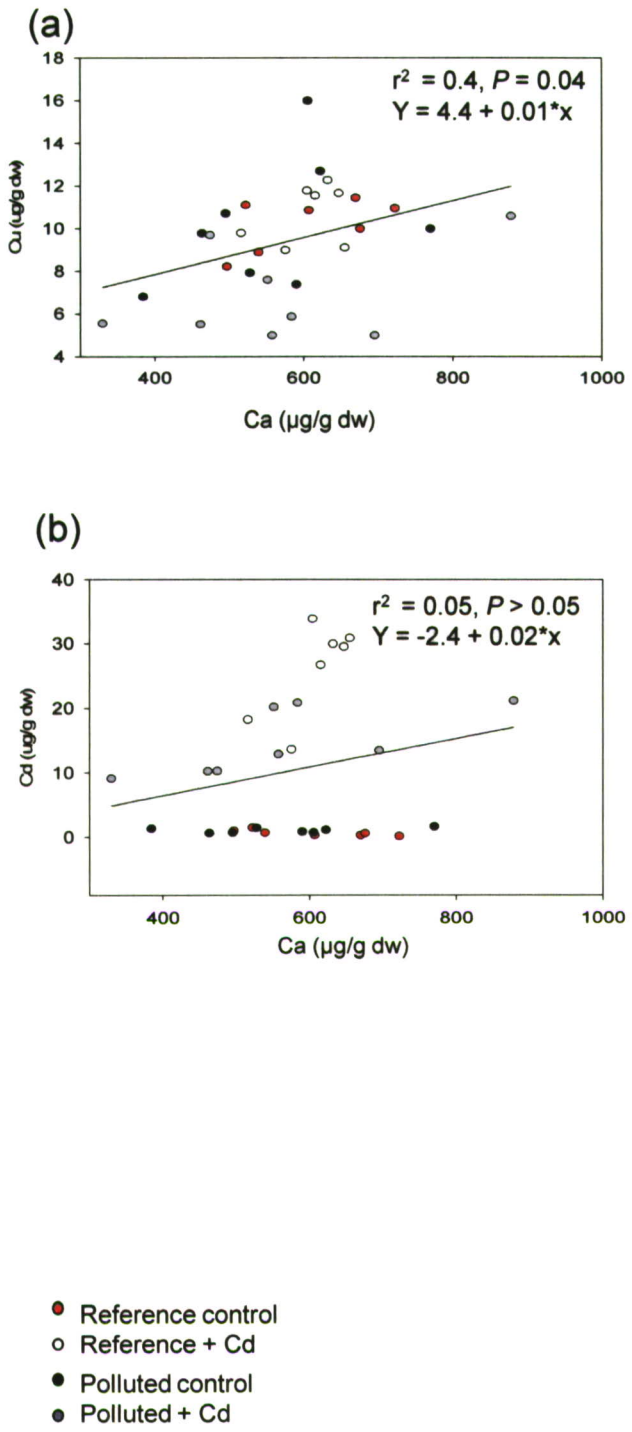

Fig. 6.8 Regression correlations illustrating Ca concentration and (a) Cu concentration, (linear fit) (b) Cd concentration (linear fit) of reference control, reference + Cd, polluted control, and polluted + Cd mussels. $n = 6 - 9$.

6.3.2 Immunological responses to cadmium exposure

Neutral red uptake and phagocytosis activity in haemocytes of mussels were measured after 24 h and on day 4 of the experiment. Neutral red uptake of mussels haemocytes was unaffected by Cd treatment (Fig. 6.9a), except a peak rise in neutral red uptake by the reference + Cd group over the polluted control site mussels at 24 h (ANOVA, $P = 0.05$). Also, haemocytes of mussels collected from the polluted site, and polluted and exposed to Cd treatment exhibited lower NRR when compared to the mussels' reference site on day 4 (ANOVA, $P = 0.01$) with significant increase in neutral red uptake in the polluted + Cd compared with the control polluted mussels. A significant time-dependent increase in neutral red uptake was observed at 4 days in the reference control mussels (ANOVA, $P = 0.003$). Two-way ANOVA was analysed on day 4 and recorded a significant difference in site × treatment ($P = 0.003$) for neutral red uptake.

At 24 h, the control polluted and polluted + Cd groups had significantly lower phagocytosis abilities compared with the reference control and reference + Cd groups (Kruskal Wallis, $P = 0.002$) (Fig. 6.9b). This indicated a strong site effect on the phagocytosis process of mussels from the polluted environment. However, the phagocytosis ability of haemocytes improved for the mussels collected from the polluted site and the difference from the reference control site mussels disappeared (ANOVA, $P = 0.5$). A two-way ANOVA on data from day 4 recorded no significant difference in site × treatment ($P = 0.9$) for the phagocytosis ability of haemocytes.

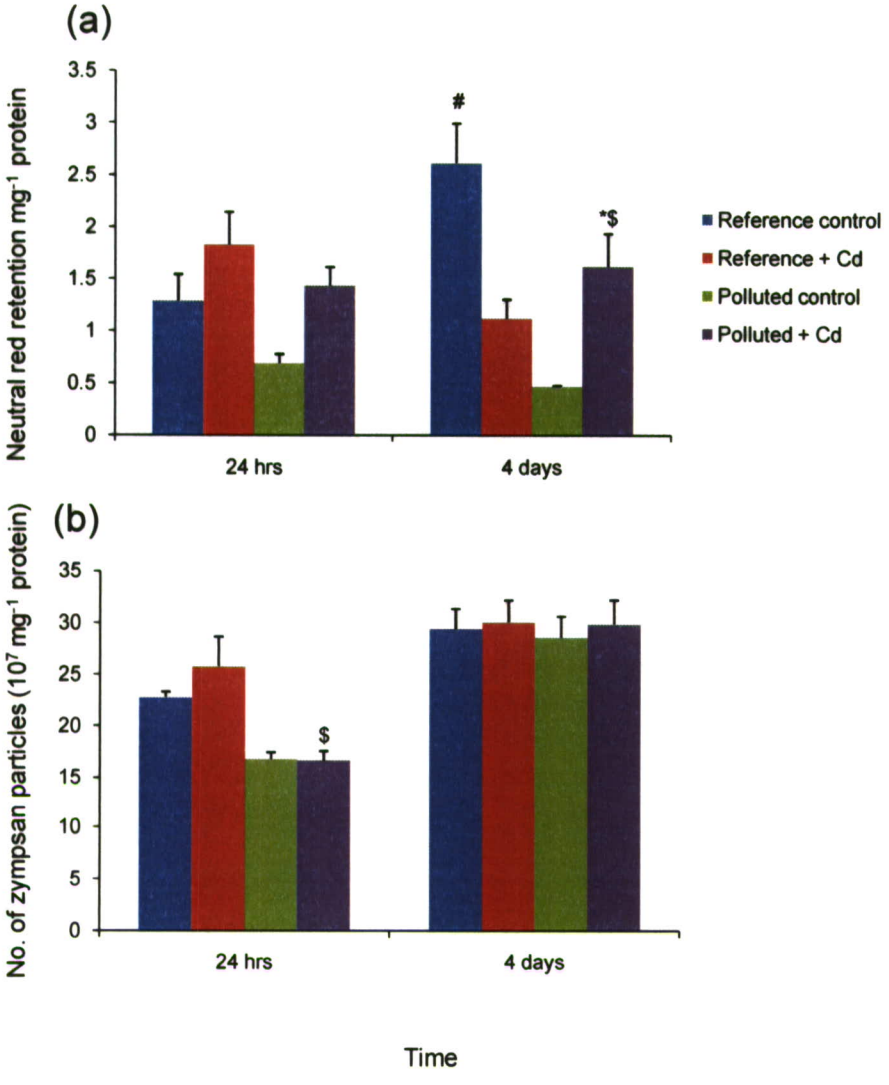

Fig. 6.9 Neutral Red uptake (a) and Phagocytosis activity (b) measured at 550 nm in *M. edulis* reference control, reference + Cd, polluted control and polluted + Cd. Data are means ± S.E.M., n = 9 per treatment at each time point. * indicates a significant difference from the respective control within field site. $ indicates a significant difference in Cd effect between sites. # indicates a significant difference in time within treatment, $P \leq 0.05$, ANOVA or Kruskal Wallis.

A haemocytes count was done at 8 days of the experiment. No significant difference was found when comparing the reference site mussels and other groups (ANOVA, $P = 0.6$). The values for the haemocyte counts were 0.8 ± 0.2, 1.2 ± 0.3, 1.0 ± 0.2, and 0.8 ± 0.2 x10^6 cells/ml for reference control, reference + Cd, polluted control and polluted + Cd, respectively. A two-way ANOVA on day 8 recorded no significant difference in site × treatment ($P = 0.2$).

6.3.3 Haemolymph and tissue osmoregulation during cadmium exposure

A significant reduction was observed in haemolymph Na concentration only on day 4 in reference + Cd treated mussels compared to reference control site mussels (ANOVA, $P = 0.01$). However, Cd treatment and pollution did not show any effect on Na regulation in haemolymph at other time points (ANOVA, $P > 0.05$). Two-way ANOVA on day 8 of haemolymph Na recorded no significant difference in site × treatment ($P = 0.7$) (Fig. 6.10a).

By contrast, K showed significantly decreased values in the polluted + Cd treatment at day 4 compared to reference + Cd animals (ANOVA, $P = 0.00001$). But, increased haemolymph K values in reference + Cd group compared to reference control mussels was recorded on day 8 (Kruskal Wallis, $P = 0.05$). A time-effect was observed for haemolymph K on day 8 for the polluted + Cd treated mussels (ANOVA, $P = 0.00001$) (Fig. 6.10b). Analysis using two-way ANOVA on day 8 recorded significant difference of haemolymph K in site × treatment ($P = 0.04$).

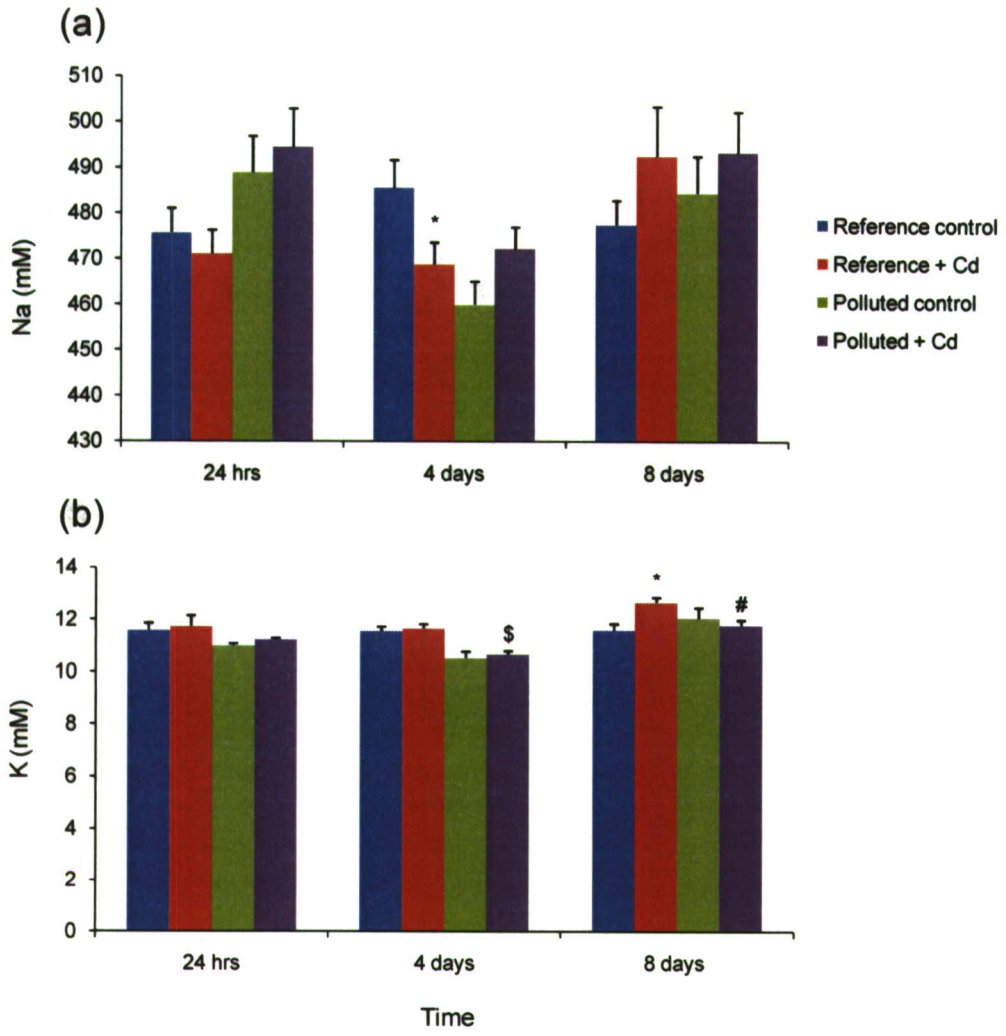

Fig. 6.10 Na (a) and K (b) concentration in *Mytilus edulis* haemolymph, reference control, reference + Cd, polluted control and polluted + Cd. Data are means ± S.E.M., n = 9 per treatment at each time point. * indicates a significant difference from the respective control within field site. # indicates a significant difference in time within treatment. $ indicates a significant difference Cd effect between sites, $P \leq 0.05$, ANOVA or Kruskal Wallis.

The gills recorded the highest organ in Na content; higher than the digestive gland or gonads (Fig. 6.11a), but with no significant difference between experimental groups on day 8, and gonads Na concentration followed the same trend. A significant increase was observed in digestive gland Na concentration only of polluted + Cd treated mussels compared with the reference + Cd and polluted control site mussels (ANOVA, $P = 0.05$ or less). Cd treatment did not show any effect on Na regulation in the digestive gland and gonads, but a site effect was apparent. No significant interaction in Na content of gills of site × treatment was found between mussels groups on day 8 after using two-way ANOVA ($P = 0.9$).

A significant increase was observed in digestive gland K levels in polluted + Cd mussels compared to reference + Cd mussels (ANOVA , $P = 0.0001$). The case was in contrast in the gills, where a significant decrease was recorded in the mussels from polluted + Cd tanks when compared to mussels from reference + Cd and polluted control animals. K content of the gonads did not show significant differences between experimental groups on day 8, which indicate no treatment or site effect (Fig. 6.11b). A significant site × treatment dependant manner was found between mussels groups in K content of gills, using two-way ANOVA ($P = 0.04$).

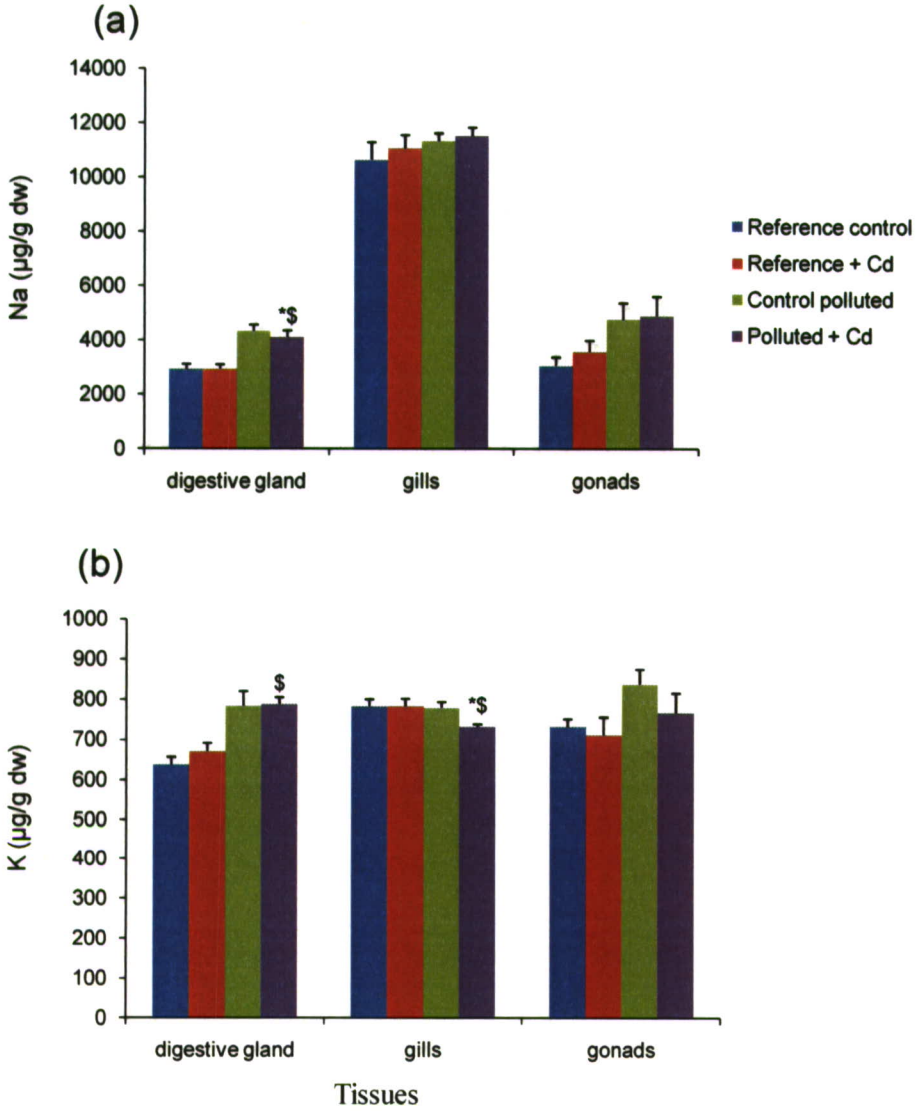

Fig. 6.11 Na (a) and K (b) concentrations in *Mytilus edulis* tissues, reference control, reference + Cd, polluted control and polluted + Cd. Data are means ± S.E.M., n = 9 per treatment at each time point. * indicates a significant difference from the respective control within field site. $ indicates a significant difference in Cd effect between sites, $P \leq 0.05$, ANOVA or Kruskal-Wallis.

6.3.4 Condition index (CI)

CI was measured at 1, 4 and 8 days of the experiment (Fig. 6.12). At 24 h, CI of the polluted + Cd mussels was significantly less when compared to all other groups (approx. 22 % less, Kruskal Wallis, $P = 0.01$). For the rest of the time points, CI lost any significant difference between treatments (ANOVA, Kruskal Wallis, $P > 0.05$). Values at the end of experiment on day 8 were 59.4 ± 1.8 to 52.4 ± 3.8, 50.2 ± 2.2 and 56.8 ± 4.0, for reference control, reference + Cd, polluted control and polluted + Cd, respectively. Analysis on day 8 revealed significant interaction in site × treatment (two-way ANOVA, $P = 0.04$). Morphometrics measurements like total body and flesh weight, shell length and weight recorded no statistical significance between sites or Cd treatment in all investigated groups (ANOVA, Kruskal Wallis, $P > 0.05$).

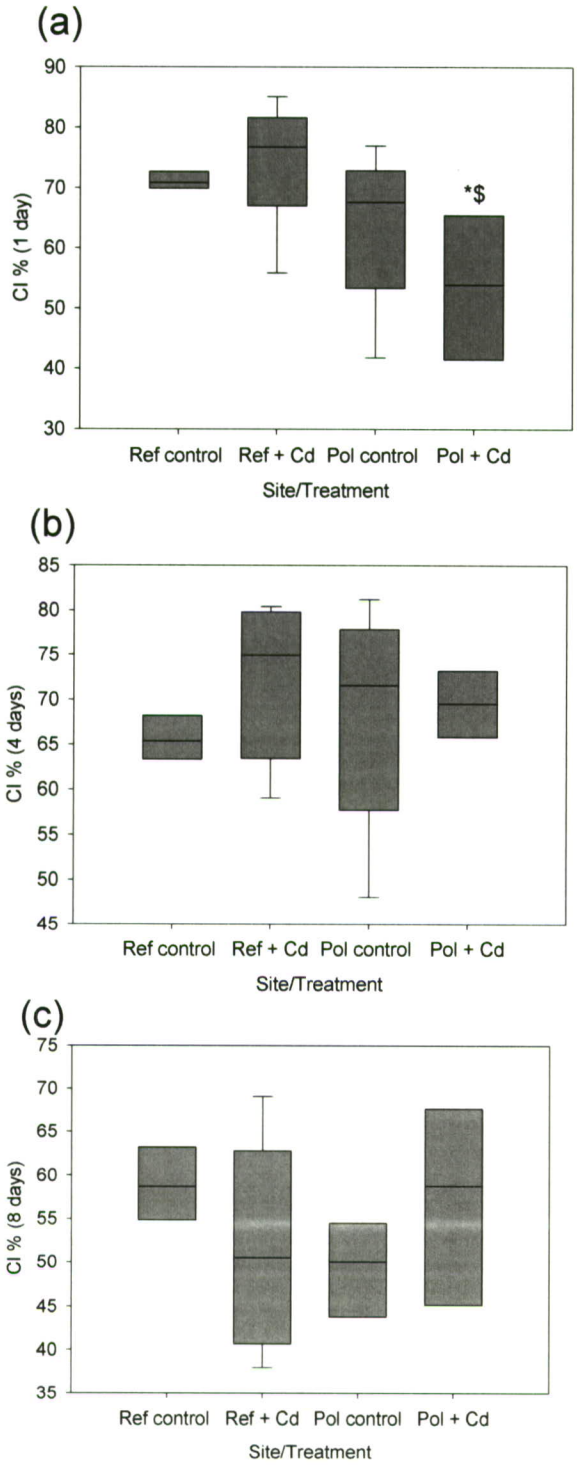

Fig. 6.12 Condition index (%) measurements at 1 day (a), 4 days (b), and 8 days (c) of reference control, reference + Cd, polluted control and polluted + Cd, n = 8 - 9 per site/treatment. $ indicates a significant difference in Cd effect between sites. * indicates a significant difference from the other groups, $P \leq 0.05$, ANOVA or Kruskal Wallis.

6.3.5 Histological alterations during Cd exposure of reference and polluted sites

6.3.5.1 Digestive gland

The reference control sections did not record any histological abnormality like necrosis or tissue atrophy (Fig. 6.13). Total injury and necrosis between groups did not show significant differences. However, digestive tubules of mussels from the polluted site showed abnormalities like necrosis of the epithelia, thinner epithelium than reference site mussels (approximately 52 % thinner) (ANOVA, P = 0.001). An inflammation-like reaction of haemocytes infiltration in the connective tissue was evident in reference + Cd mussels. The reference + Cd mussels recorded necrosis, but with a thicker epithelium compared to animals from the polluted control site (ANOVA, P = 0.0007). Haemocytes infiltration in the connective tissue was recorded in all sections examined, but granulocytoma in one section only (Fig. 6.14b). All polluted + Cd mussels showed alteration in the digestive gland, especially the digestive tubules. The digestive tubules of polluted treated mussels showed similar symptoms like a thinner epithelium when compared to reference control (approximately 46 % thinner) and reference + 20 μgl^{-1} Cd (approximately 36 % thinner) groups (ANOVA, P = 0.001) and foci of inflammation (Fig 6.14). The thin epithelium of digestive tubules was a syndrome of pollution, because it related only significantly to site effect (see Table 6.1).

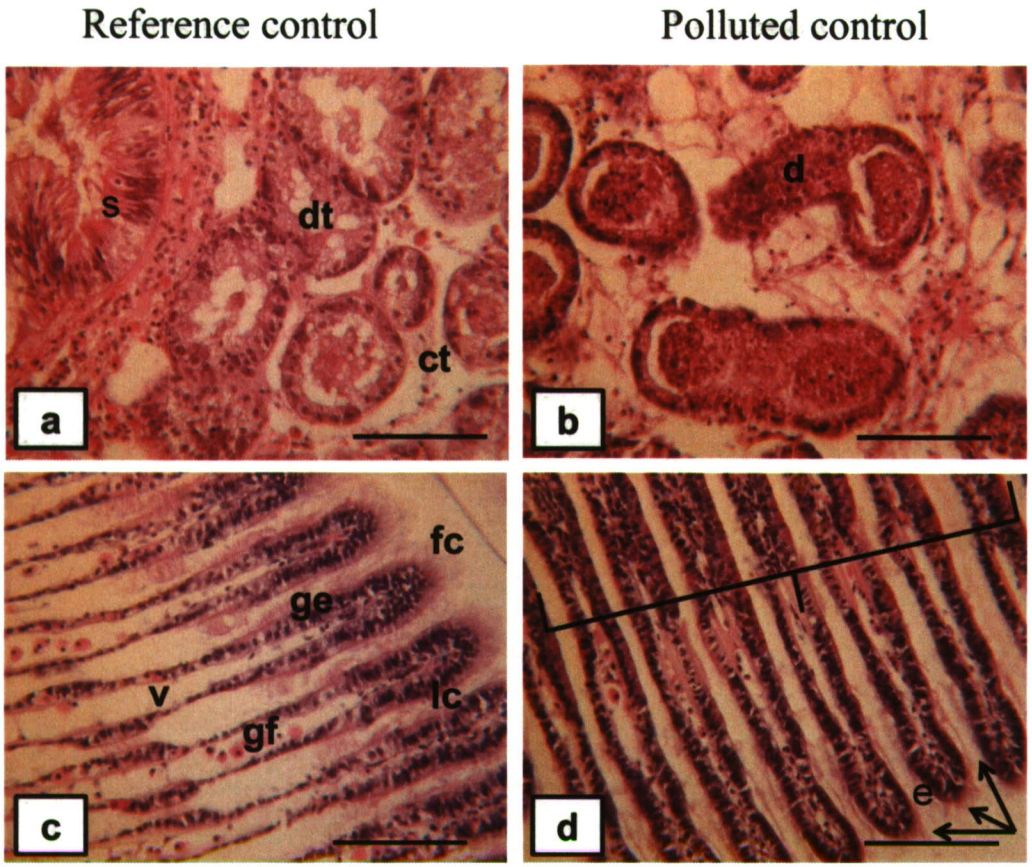

Fig. 6.13 Light micrographs of sections through digestive gland and gills of *M. edulis* showing histological structure of reference control and polluted control mussels on day 8, stained with Haematoxylin and Eosin at 5-10 μm thickness. (a and c) digestive gland and gills of reference control mussels, (b and d) digestive gland and gills of polluted control mussels, with degenerated digestive tubules and eroded cilia from gill filaments and blocked haemolymph vessels (bracket). ct, connective tissue; s, stomach; d, degeneration; e, erosion; gf, gill filaments; fc, frontal cilia; lc, lateral cilia; and ge, gill epithelium; and v, haemolymph vessel. Scale bar, 100 μm.

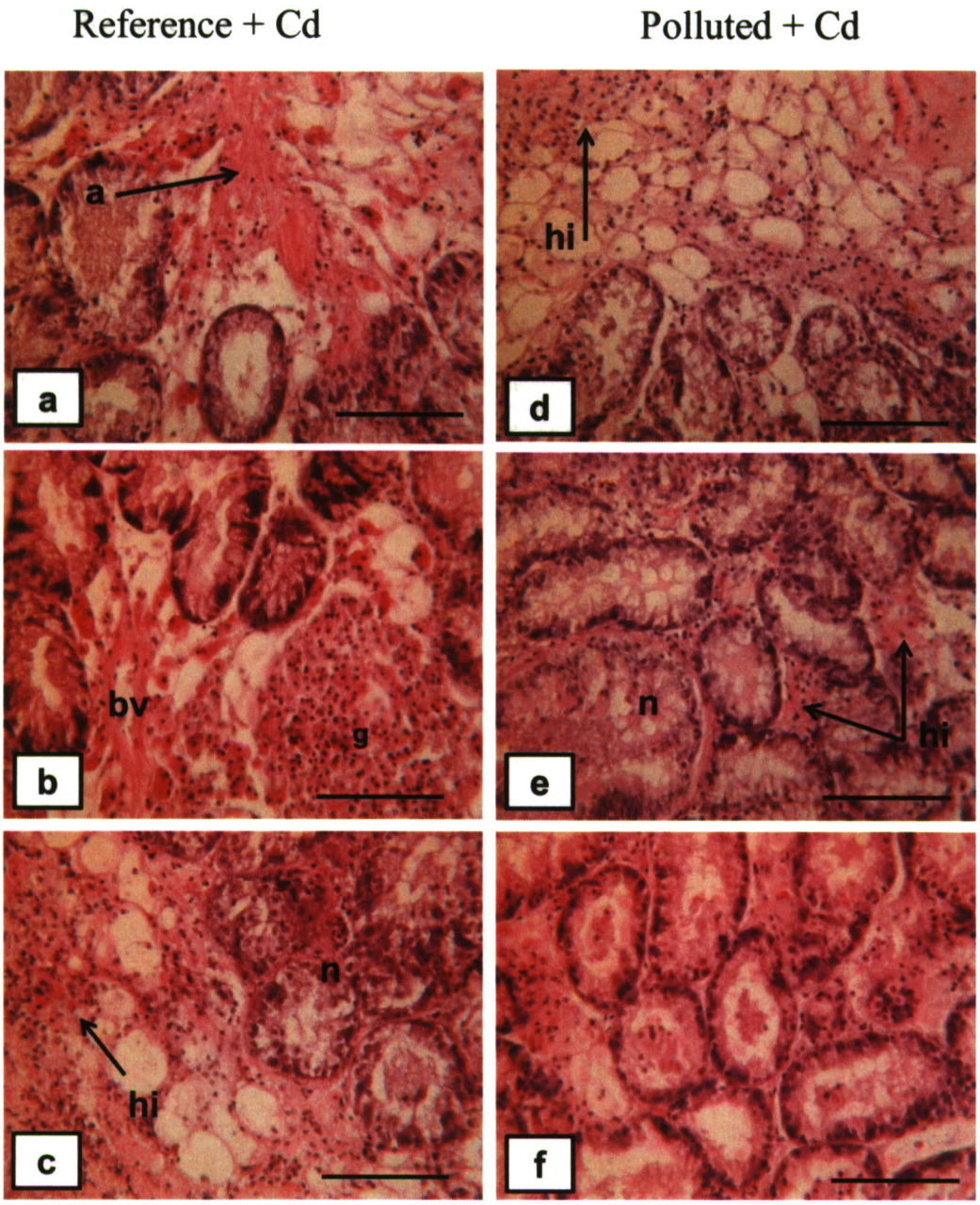

Fig. 6.14 Light micrographs of sections through digestive gland of *M. edulis* showing histological structure of reference + Cd and polluted + Cd mussels on day 8, stained with Haematoxylin and Eosin at 5-10 μm thickness. (a), (b) and (c) reference + 20 μg l^{-1} $CdCl_2$ mussels with necrosis, granulocytoma and connective tissue atrophy; (d), (e) and (f) polluted + 20 μg l^{-1} $CdCl_2$ mussels with necrosis and haemocytes infiltration. n, necrosis; hi, haemocytes infiltration; g, granulosytoma; a, atrophy and bv, blood vessel. Scale bar, 100 μm.

Table 6.1 Quantitative histological analysis of digestive gland in *M. edulis* reference control, polluted control, reference + Cd and polluted + Cd mussels on day 8.

Treatment	Fractional area of tubule	Maximum tubule diameter (μm)	Epithelium diameter (μm)	Injured tubules (%)	Necrotic tubules (%)	Inflammation
Reference control	69.5 ± 6.3	121.9 ± 5.8	59.9 ± 6.3	23.1 ± 0	23.1 ± 0	-
Reference + Cd	66.7 ± 8.3	114.7 ± 4.3	50.6 ± 3.1	55.7 ± 0.7	50.8 ± 0.6	+++
Polluted control	64.5 ± 8.8	105.9 ± 7.5	28.6 ± 7.6	59.5 ± 1.1	57.9 ± 1.1	+
Polluted + Cd	66.4 ± 12	97.4 ± 6.1	32.2 ± 2.1 $	50.1 ± 1.7	50.1 ± 1.7	++

Data are means ± S.E.M., $n = 9$ mussels per treatment. $ indicates a significant difference in Cd effect between sites, ANOVA or Kruskal Wallis when $P \leq 0.05$. Note. – indicates absent inflammation, + rare inflammation, ++ moderate inflammation and +++ frequent inflammation.

6.3.5.2 Gills

Almost all gills from reference controls showed normal histology without symptoms of necrosis or filaments with atrophy (Fig. 6.13). All other groups, other than the reference control, exhibited highly significant increases in gill injury (ANOVA, P = 0.00001). Gill filaments injury was 4, 3.2 and 3.9 times greater in control polluted, reference + Cd and polluted + Cd mussels than the reference control site mussels on day 8. The polluted mussels exhibited necrosis in the epithelia (approximately 1 fold increase than reference + Cd mussels), erosion of the frontal cilia, but not obvious inflammation. Mussel groups from polluted + 20 μgl^{-1} Cd showed significant increases in necrotic epithelia on the filaments when compared to reference site animals (ANOVA, P = 0.02) and approximately 2 fold increase compared to polluted + Cd mussels. Mussels from the polluted site + Cd treatment revealed similar pathological symptoms as the reference + Cd treated ones. These symptoms which appeared in polluted + Cd mussels are not statistically different in necrotic epithelia compared to any of other groups, and inflammatory reactions doubled in the haemolymph vessels compared with all other groups (Fig. 6.15, Table 6.2).

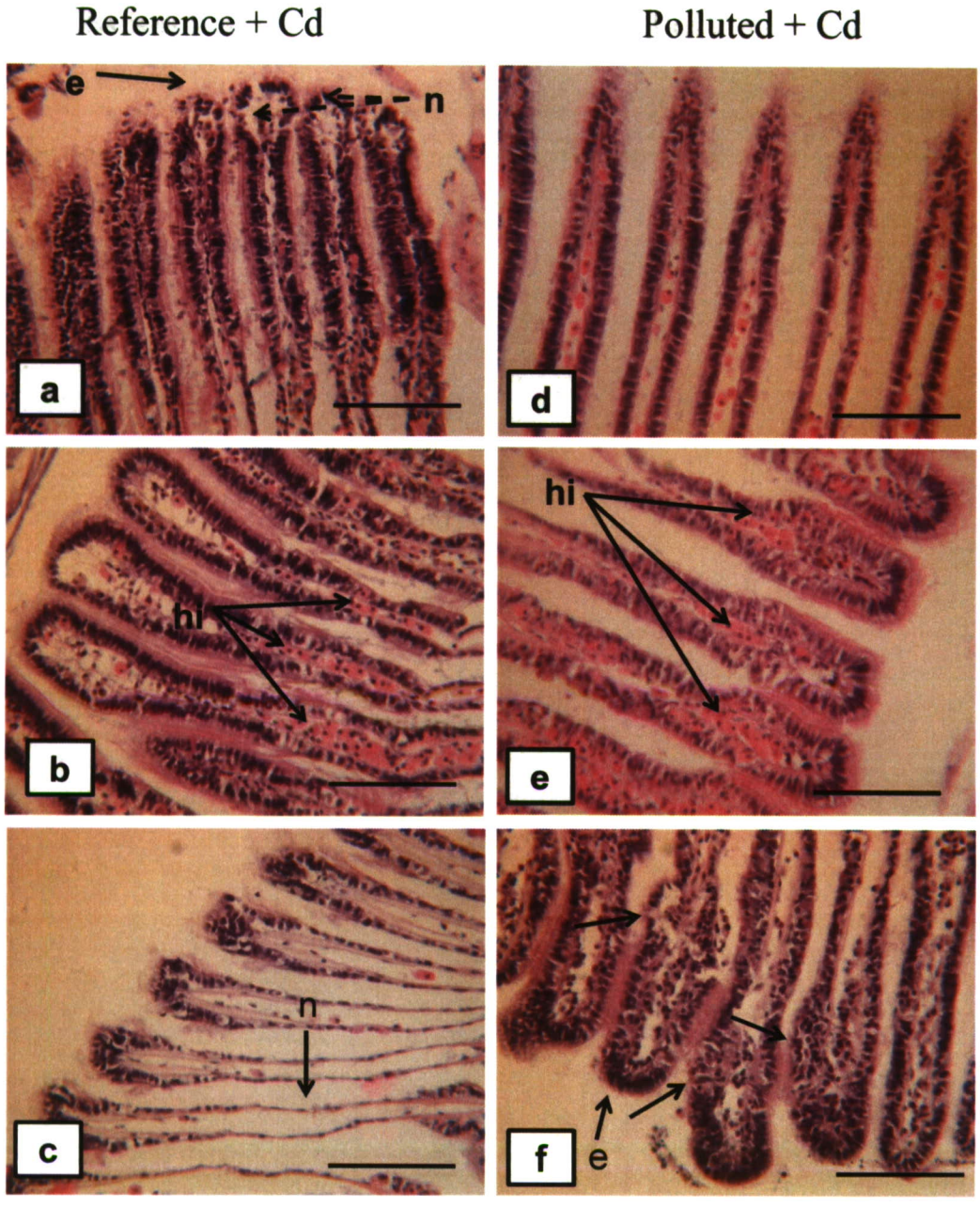

Fig. 6.15 Light micrographs of sections through gills of *M. edulis* showing histological structure of reference + Cd and polluted + Cd mussels on day 8, stained with Haematoxylin and Eosin at 5-10 µm thickness. (a), (b) and (c) reference + 20 µg l^{-1} $CdCl_2$ mussels, (d), (e) and (f) polluted + 20 µg l^{-1} $CdCl_2$ mussels. All mussels groups exhibited inflammation, epithelial necrosis, gill atrophy and erosion of the frontal cilia. v, haemolymph vessel; n, necrosis; hi, haemocytes infiltration; e, errosion. Scale bar, 100µm.

Table 6.2 Quantitative histological analysis of gill in *M. edulis* collected from reference control, polluted control, reference + Cd and polluted + Cd mussels on day 8

Treatment	No. of filaments	Inflammation	Injury of filaments (%)	Necrotic filaments (%)
Reference control	5.3 ± 0.4	+	17.1 ± 0.3	0.0 ± 0.0
Reference + Cd	6.1 ± 0.3	+	54.1 ± 0.4 *	43.8 ± 0.7 *
Polluted control	5.9 ± 0.4	+	73.8 ± 0.6	58.6 ± 1.1
Polluted + Cd	5.5 ± 0.3	++	67.1 ± 0.7	19.6 ± 0.7

Data are means ± S.E.M., n = 9 mussels per treatment. * indicates significant difference from the respective control within field site, ANOVA or Kruskal Wallis when $P \leq 0.05$. Note. + indicates rare inflammation, and ++ moderate inflammation.

6.3.5.3 Gonads

The male gonads of control animals showed normal architecture, with well defined spermatic follicles and surrounding connective tissue. The sperm tails appear in the middle of the follicle, and the spermatogonia surrounding the peripheral of it (Fig. 6.16). Total injury increased significantly in all groups compared with reference control mussels (ANOVA, P = 0.004). Mussels from the polluted control site had tissue inflammation and necrosis plus spermatogonia degeneration. Decreased fractional area of spermatic follicles to connective tissue area is site effect, where polluted control and polluted + Cd groups showed significant decreased follicles area compared to reference control and reference + Cd mussels (ANOVA, P = 0.0009). The spermatic follicles area in polluted control showed 76 % decrease compared to reference control mussels, and polluted + Cd mussels recorded 37 % decrease when compared to the reference control mussels, (Table 6.3). Necrosis is a site effect in the polluted control and polluted + Cd groups compared to the reference control (ANOVA, P = 0.004), and to a lesser extent in reference + 20 $\mu g\ l^{-1}$ $CdCl_2$ group compared to the reference control animals as a treatment effect (ANOVA, P = 0.004) (Table 6.3). The polluted + 20 $\mu g\ l^{-1}$ $CdCl_2$ showed similar symptoms as the reference + Cd treated mussels only but more severe as more necrosis recorded and less inflammatory reactions appeared (Fig. 6.17 d, e and f).

The female gonads of control animals showed normal architecture, with well defined ovarian follicles and surrounding connective tissue. Several oocytes filled in the ovarian follicle, and the oogonia surrounding the peripheral of it (Fig. 6.16). The polluted mussels were characterized by increased degeneration of the oocytes inside the ovarian follicle compared to the reference control one. Reference animals exposed to the 20 $\mu g\ l^{-1}$ Cd treatment showed degenerative ovarian follicles, significantly reduced fractional area of egg follicles (approximately half area or more of other groups) and had a heavy haemocytes infiltration; double that of all other groups (Table 6.3).

A severe inflammatory reaction was recorded in one of the ovarian follicles examined (Fig. 6.18b). The polluted + 20 μg l^{-1} $CdCl_2$ mussels showed different symptoms compared to the reference + Cd ones like empty and degenerative/necrotic ovarian follicles that were filled with cellular debris (Fig. 6.18d, e and f). Necrotic follicles increased significantly in all groups when compared to reference control site gonads (ANOVA, $P = 0.001$) (Table 6.3).

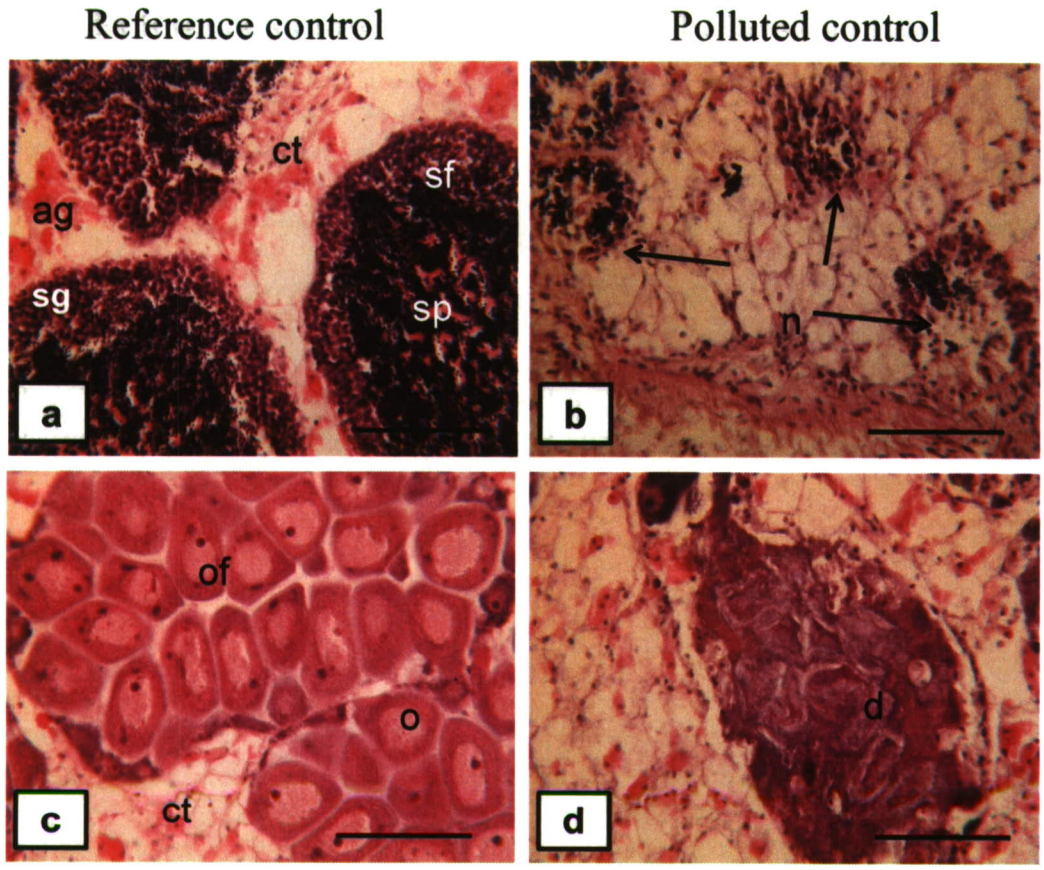

Fig. 6.16 Light micrographs of sections through male and female gonads of *M. edulis* showing histological structure of reference control and polluted control mussels on day 8, stained with Haematoxylin and Eosin at 5-10 µm thickness. (a and c) male and female gonads of reference control mussels, (b and d) male and female gonads of polluted control mussels, with necrotic spermatic follicles (arrows) and degenerated ovarian follicles. ct, connective tissue; n, necrosis; of, ovarian follicle; o, oocyte; sf, spermatic follicle; sp, sperm flagella; ag, adipogranular cells; d, degeneration and sg, spermatogonia. Scale bar, 100 µm.

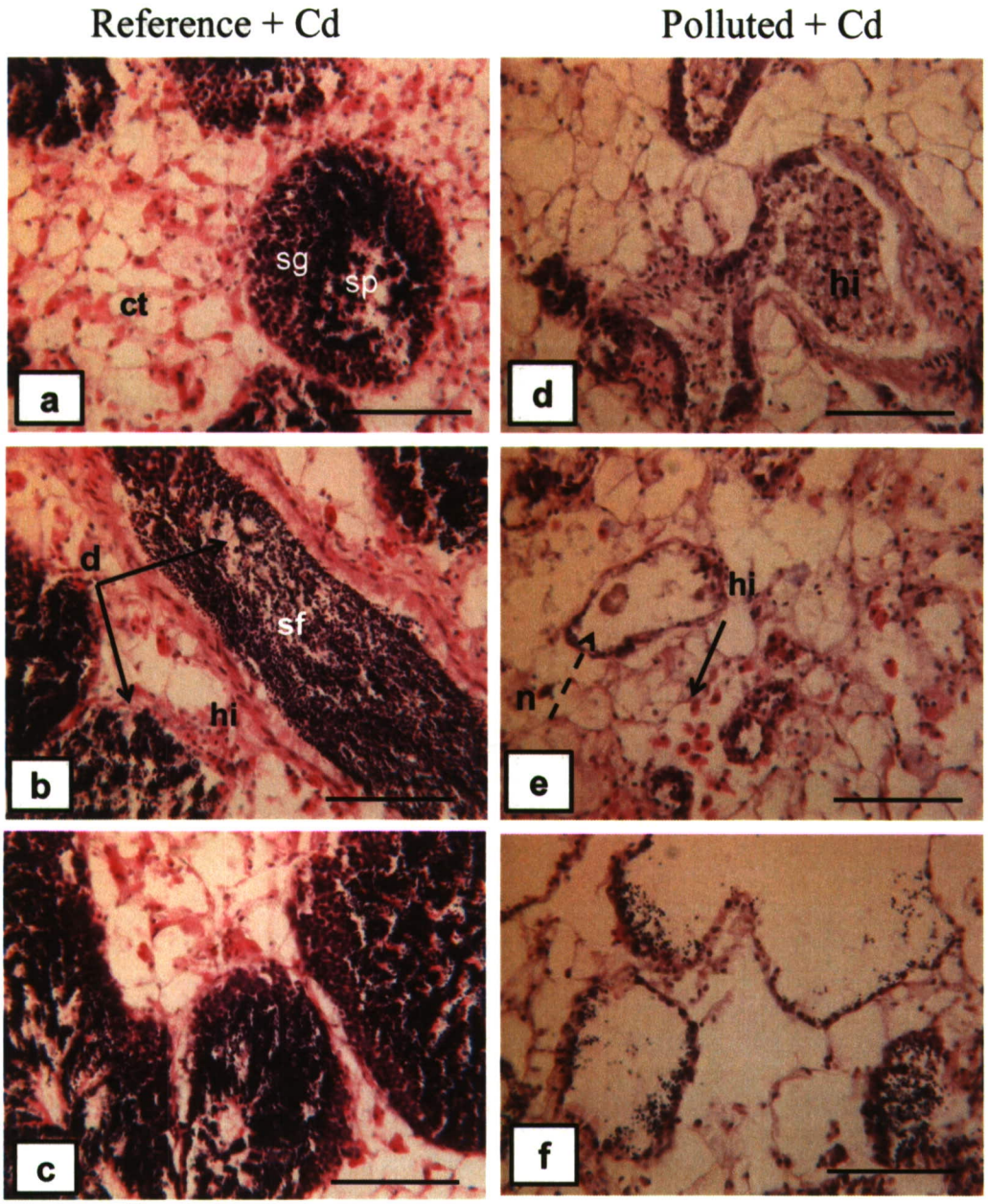

Fig 6.17 Light micrographs of sections through male gonads of *M. edulis* showing histological structure of reference + Cd and polluted + Cd treated mussels on day 8, stained with Haematoxylin and Eosin at 5-10 μm thickness. (a), (b) and (c) reference + 20 μg l^{-1} $CdCl_2$ mussels, (d), (e) and (f) polluted + 20 μg l^{-1} $CdCl_2$ mussels. Mussels from both groups showed inflammation, necrotic spermatic follicles and degeneration. n, necrosis; sf, spermatic follicle; sp, sperm flagella; sg, spermatogonia; ct, connective tissue; and hi, haemocytes infiltration. Scale bar, 100 μm.

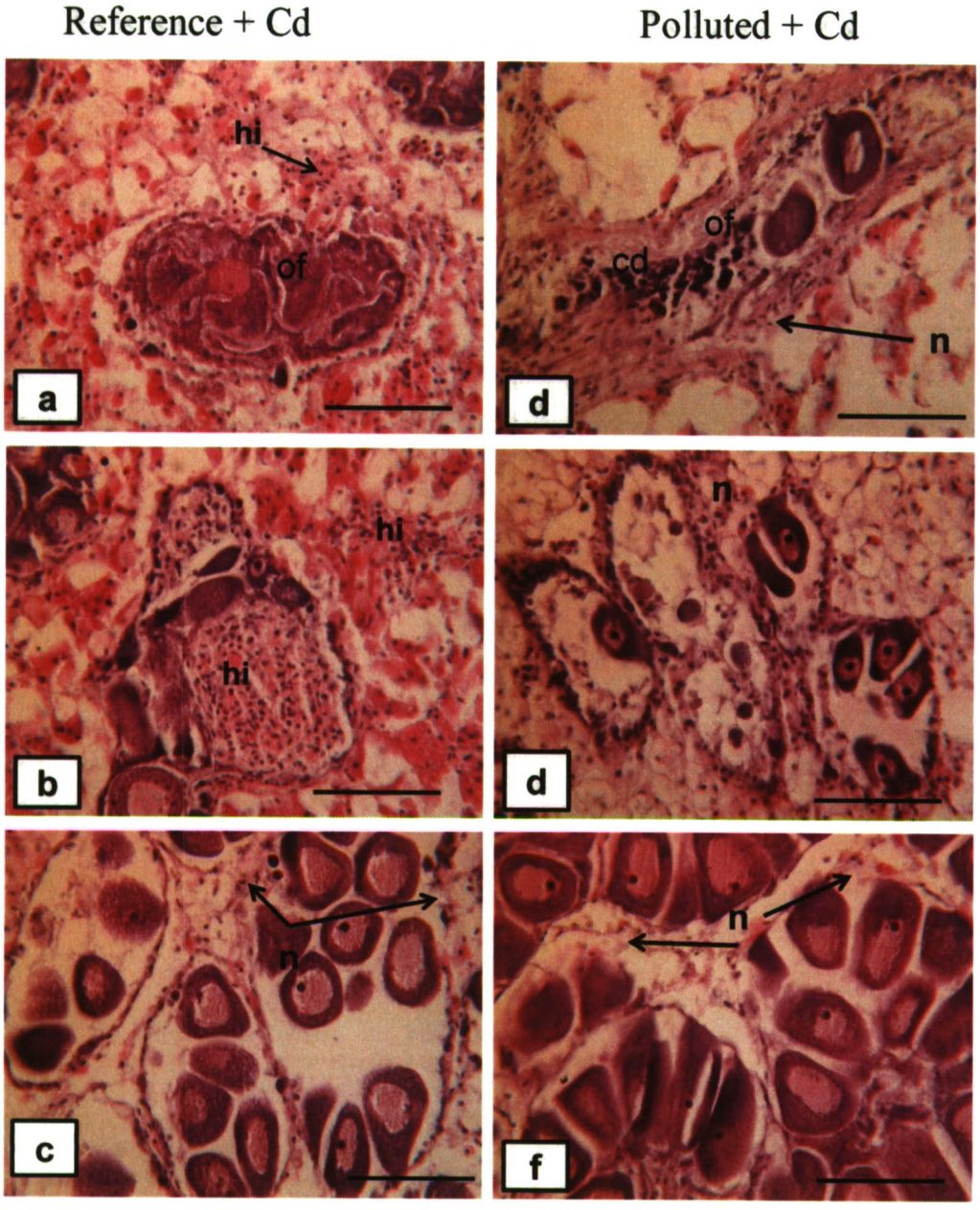

Fig. 6.18 Light micrographs of sections through female gonads of *M. edulis* showing histological structure of reference + Cd and polluted + Cd mussels stained with Haematoxylin and Eosin at 5-10 µm thickness. (a), (b) and (c) reference + 20 µg l^{-1} $CdCl_2$ mussels exhibited inflammation and degeneration, (d), (e) and (f) polluted + Cd mussels with necrotic follicles, cellular debris and degenerative oocytes. of, ovarian follicle; n, necrosis; hi, haemocytes infiltration; and cd, cellular debris. Scale bar, 100 µm.

Table 6.3 Quantitative histological analysis of gonads (male and female) injury in *M. edulis* reference control, polluted control, reference + Cd and polluted + Cd mussels on day 8.

Gonads	Treatment	Fractional area of spermatic/egg follicles	Maximum oocyte diameter (μm)	Nuclear material diameter of oocyte (μm)	No. of injured spermatic/egg follicles (%)	Necrotic follicles (%)	Inflammation
Male	Reference control	67.8 ± 6.7			0.7 ± 0.3	0.0 ± 0.0	+
	Reference + Cd	59.7 ± 10.7			1.6 ± 0.0 *	72.2 ± 0.5 *	+++
	polluted control	22.2 ± 5.3			1.6 ± 0.2	99.5 ± 0.5	++
	polluted + Cd	37.8 ± 8.4 $			2.0 ± 0.0	99.6 ± 0.4 $	++
Female	Reference control	81.1 ± 5.0	64.4 ± 1.7	34.7 ± 3.1	0.0 ± 0.0	0.0 ± 0.0	-
	Reference + Cd	34.6 ± 4.0 *	62.0 ± 0.0	36.6 ± 0.0	2.0 ± 0.0 *	78.8 ± 0.2 *	++
	polluted control	63.9 ± 11.2	58.8 ± 7.9	33.4 ± 6.4	2.0 ± 0.0	52.8 ± 0.3	+
	polluted + Cd	67.8 ± 12.4 $	62.0 ± 9.5	31.8 ± 9.5	1.7 ± 0.3	77.8 ± 0.3	+

Data are means ± S.E.M., $n = 9$ mussels per treatment. * indicates a significant difference from the respective control within field site. $ indicates a significant difference in Cd effect between sites, ANOVA or Kruskl-Wallis when $P \leq 0.05$. Note. - indicates absent inflammation, + rare inflammation, ++ moderate inflammation, and +++ frequent inflammation.

6.4 Discussion

Many studies on bivalves collected from polluted sites report modulation of immunity, physiology, and histology (e.g., Pickwell and Steinert, 1984; Auffet, 1988; Wedderburn, 2000; Galloway et al., 2002; David et al., 2008b) as an effect of pollutants. Cadmium accumulation and the induction of MT proteins has been broadly studied on marine bivalves (Soazing and Marc, 2003; Lecoeur et al. 2004; Fasulo et al. 2008). However, this study is one of the first experiments on *M. edulis* to evaluate pre-exposure history followed by Cd treatment on metal accumulation, cellular immunity, CI, osmotic balance, *mt10* gene expression (Chapter 7) and histopathology in the same study. This study recorded transient changes in immunological functions of haemocytes during exposure, adverse tissue pathologies, but counter intuitively, Cd treatment also improved symptoms of the pre-exposure history effects in some of the study parameters.

6.4.1 Metal accumulation during cadmium exposure

Cadmium exposure was confirmed by measuring Cd accumulation in the tissues (Fig. 6.2). The background levels of Cd in the tissues of reference control mussels were as few as $\mu g\ g^{-1}$ dry weight. Cadmium levels in the tissues were depending on exposure concentrations and time dependant. The digestive gland recorded the highest organ in Cd accumulation; see Chapter 4 for more detailed discussion of Cd accumulation. We found no effect of site (exposure history) on metal distribution in tissues after Cd treatment. Similar observation had been made by Handy (1995), who recorded no difference in mercury distribution in fish tissues exposed to chronic or intermittent regimes of mercury exposure. This may mean that multiple exposures will not change accumulation of metal in the target tissue. In our experiment, we found that the non-

essential metals like Cd, Fe and Pb accumulation was higher in polluted + Cd mussels than reference + Cd mussels` tissues. A similar finding was recorded by Handy (1995), who found increased mercury concentration in fish tissues exposed to chronic concentration of $HgCl_2$ compared with those intermittently exposed to the same metal.

Calcium levels in the tissues were found to be the highest in the gills followed by the digestive gland then the gonads and finally to a lesser extent in the haemolymph (Fig. 6.3). The treatment and contamination significantly affected and reduced Ca levels in the tissues and haemolymph as well. Schoenmakers et al. (1992) reported that cadmium (IC50 of 8.2 pM) affects Ca^{2+} ATPase in tilapia intestine and as a result inhibits the calcium pump and Verbost et al. (1988) reported the consistent finding in gills (IC50 of 2 nM) where ATP-driven Ca^{2+}-transport was inhibited by Cd^{2+}. The gills recorded the highest levels of Zn and Cu accumulation from all the tissues studied (Fig. 6.4 and 6.5). This finding is consistent with Gammon et al. (2009) where the gills of the marine gastropod *Littorina littorea* collected from Plymouth Sound, UK had high Zn and Cu accumulation. In our study, treatment with Cd has no effect on the copper accumulation in the tissues or the haemolymph, but exposure history of pollutants affected Cd, Fe, Zn and Cu accumulation in mussels' tissues more than Cd exposure alone (Figs. 6.2 - 6.6). Comparable findings observed in a study on clams (*Macoma balthica*) from a contaminated area, mussels accumulated less Cu in their tissues than mussels from clean areas when they were both exposed to 30 or 40 $\mu g\ l^{-1}$ Cu for 13 days. This is because the former can regulate metal accumulation in their tissue as they are able to limit metal accumulation (Ballan-Dufrancais et al., 2001). Fe in our study was found to accumulate mainly in the gills (Fig. 6.6). A similar finding was recorded by Canli and Atli (2003) in six Mediterranean fish species. Pb levels in gills and haemolymph followed the same pattern where only polluted control mussels were the highest in accumulation and no exposure effect on Pb concentrations but site effect is

approved (Fig. 6.7). Odzak et al. (1994) found no interaction between Pb and Cd exposure in *M. galloprovincialis* gills after exposure to both metals for seven days.

6.4.2 Effect of pollution and cadmium on haemolymph and immune functions of haemocytes

Neutral red content of haemocytes decreased significantly on day 4 from polluted control mussels (Fig. 6.9a). In the present work, mussels experienced contamination stress at the beginning of the experiment then recovered. Chronic exposure to pollutants had a modulating effect on haemocytes immunfunctions, neutral red uptake and phagocytosis. Pipe and Coles, (1995) exposed mussels to up to 7 weeks of Cu followed by bacterial challenge. They found an inhibition effect of exposures history on some parameters as haemocytes counts and stimulation in others like superoxide production. Also, Hagger et al. (2010) argued that neutral red retention time seasonal variability could be reduced as a result of pollution if the organism is already at the extreme range of its tolerance capabilities. In our study, a significant inhibition effect of chronic exposure in the field + Cd exposure in the laboratory was recorded on the phagocytosis ability of haemocytes compared to Cd exposure only (Fig. 6.9b). Also, on day 8, the haemocytes count recorded a decrease in the number of polluted + Cd mussels when compared to reference + Cd mussels. Sokolova et al. (2004) found that increased Cd concentrations inversely decrease ATP content of the haemocytes of *Crassostrea virginica* oyster. From above we can conclude that pollution compromises the energy sources of the haemocytes and indirectly affects the phagocytosis process as it is an energy-dependant function as animals from polluted sites always suffer from food shortage (Luengen et al., 2004).

Haemocyte count data showed that it is affected by pollution and Cd exposure, that they decreased in the polluted + Cd treatment and increased in reference + Cd mussels but not significantly. The total increase in the number of circulating haemocytes, after exposure to a pollutant, could be a result of (1) haemocytes moving from tissues into the circulation, or (2) haemocyte proliferation (Pipe and Coles, 1995). Again, instead of decreased haemocytes count, may be more cells penetrate tissue to repair tissue injury like inflammatory reactions in polluted + Cd mussels and more proliferation of haemocytes in the circulation like the case of reference + 20 $\mu g\ l^{-1}$ Cd mussels as proved in tissue inflammation by histology examination. Ford et al. (1993) pointed out similar ideas in oysters infected with the protozoan parasite *Haplosporidium nelsoni.* Haemocyte counts had higher values in the reference + Cd and polluted control mussels than the reference control at the end of the experiment. This is in agreement with Fisher et al. (2000) who found a general increase in immune responses (haemocytes density) with increased xenobiotic chemicals in tissues of contaminated areas. An additional reason for increased circulating haemocytes of exposed mussels to pollutant is given by Baier-Anderson and Anderson (2000), as the attached haemocytes in the lining of the circulatory system move to the stream of circulation under exposure.

Osmoregulation is one of the gills' functions, and its injury appeared to compromise haemolymph electrolytes. Disturbance in ionic balance can dramatically affect the vital physiological functions of the organism. There were significant effects of Cd exposure on haemolymph electrolyte levels (Fig. 6.10). The ionic composition of bivalve haemolymph always follows the external medium they live in, but some disturbance had happened to our exposed mussels on day 4. In this study, Na and K decreased significantly on day 4 of polluted + Cd and polluted control mussels. The findings are in agreement with Larsson et al. (1981) who studied the effect of sublethal cadmium levels (5-500 mu. g Cd/l) on the flatfish *Platichthys flesus* L in brackish water

during 4 - 9 weeks and recorded a strong depression in potassium and calcium concentrations in the blood plasma.

In the present study, a good correlation was found between Ca and Cu concentration in digestive gland (Fig. 6.8). On the other hand, poor/weak correlations were found between Ca and the other essential metals like Zn and Fe, and surprisingly the non-essential metals as Cd and Pb (Fig. 6.8). Ireland and Marigomez (1992) found a trend in Cu accumulation in the snail digestive gland where increased Ca^{2+} in diet decreased the Cu concentration in tissue experimentally. It is well known that Cd uptake through the cells can be via calcium pumps because they have similar ionic radius 0.92 and 0.94Å (Williams and Frausto da Silva, 1996). However, Cu and Na can interfere and affect each other's concentration. In the present study, Na was not affected in polluted + Cd mussels` gills and significant decreased Cu concentrations in the same tissue group of mussels were recorded. Handy et al. (2002) reviewed evidence that Cu ions inhibit Na^+ K^+-ATPase and may increase [Na+]i, because Na^+ and Cu ions may share a common uptake pathway into the epithelium from the external medium.

Condition index showed a significant decrease in polluted + Cd mussels compared with the reference control and reference + Cd on 24 h and then this significance disappeared in the following time points (Fig. 6.12). Geffard et al. (2005) found an inconsistency in the trend of CI between mussels from control and transplanted sites. They also recorded organ weight increase in control mussels compared with transplanted site mussels. In an earlier study, Veldhuizen-Tsoerkan et al. (1991a) found no effect of controlled stressors (aerial exposure and temperature) on the mussels from the polluted site CI. In our exposure of only 8 days, it would also be unlikely that morphometrics would change much due to the Cd exposure, and most of the effects simply relate to the sizes of animals obtained in the field.

6.4.3 Organ pathologies from contaminant and cadmium exposure

The effect of contaminants and cadmium on the histological structure of digestive gland, gills and gonads was investigated using light microscope (Figs. 6.13 – 6.18). All the tissues showed histological alterations associated with pre-exposure history or Cd treatment.

The effects of Cd/contaminats on the digestive gland were severe all together including necrosis, thinning of the tubule and inflammation in the connective tissue spaces (Fig. 6.14, Table 6.1). Da Ros et al. (1995) also found significant thinning in the digestive tubules of *Mytilus species* in the treated groups with Cd. Similar findings to granulocytoma was reported by Auffret (1988) in the digestive gland of *M. edulis* from a contaminated Norwegian fjord site. This necrosis and inflammation in the digestive gland is likely to be an indirect effect of Cd in the tissue, since Cd levels in the digestive gland of exposed animals was much higher than the controls and the other organs. Cd is also evidently caused by significant increases of Ca^{2+} concentrations in the ventricular fluid in the freshwater bivalve *Anodonta cygnea* (Faubel et al., 2008), and loss of Ca^{2+} homeostasis is a key step in initiating cellular necrosis.

The gill sections of polluted + Cd or reference + Cd mussels showed loss of the lateral cilia (Fig. 6.15, Table 6.2), epithelial necrosis and haemocytes infiltration in the haempolymph vessels. Sunila, (1988) confirmed that symptoms like inflammation, oedema and loss of lateral cilia are common features associated with heavy metal exposure. This can be explained as the gills of bivalves are organs in direct contact with the contaminants as a result of their exposed epithelial surface (David et al., 2008a). In another study, David et al., (2008b), observed detachment of the gill epithelium, inflammatory responses and increased mucous cells number. The authors explained these changes in cell structure as a result of absorption of the unwanted toxicant (eg.,

trace metals), and a way to prevent these toxicants from being absorbed. During the current study, Cd treatment/pollution exposure caused altered testicular structure in the male gonads, characterised by inflammation, necrosis and degeneration of spermatic follicles (Fig. 6.17, Table 6.3). Aarab et al. (2004) recorded inflammation and degeneration in spermatic follicles of *M. edulis* after exposure to North Sea oils. Syiasina and Vaschinko (1996) reported quite similar pathological symptoms to ours in the male gonads of sea urchin under the effect of cadmium exposure for 130 days, or pollution in the Sea of Japan. Testes containing very few or no spermatozoa was one of the pathology detected after nonylphenol treatment of male eelpout fish (Christiansen et al., 1998). The seminiferous tubules in rats administered 80 mg of an organophosphorus pesticide for 2 months showed different appearance, reduced size and disruption of normal epithelial organization (Boockfor and Blake, 1997). The important role of granulocytes in heavy metal detoxification has been explained by Giambrini et al. (1996). Oocytes atresia is detected in fishes exposed to xenobiotic compounds, where degenerative and necrotic ova are accompanied by macrophage infiltrations (McCormick et al., 1989). So the presence of haemocytes aggregations in our examined tissues is for a reason in the tissues of the mussels from reference + Cd, polluted control and polluted + Cd mussels as a defence, detoxification and tissue repair reactions. Almost all the pathology recorded in reference + Cd and polluted + Cd mussels are the same. This means that heavy metals pollution as a mixture or single metal give very similar symptoms of pathology. However, a mixture of metals can neglect the effect of each other and single metal can has worse effects on the organism.

We also observed injury to the female gonads during Cd/contaminants exposure, characterised by inflammation, necrosis and degeneration of egg follicles (Fig. 6.18, Table 6.3). The same finding (degenerative ovarian follicles and inflammation) were recorded by Aarab et al. (2004) after *M. edulis* were exposed to North Sea oils. They

explained that a combination of pollutants can cause endocrine disruption and pathological effects. Binelli et al. (2004) reported some similar pathology in female gonads of zebra mussels at field sites contaminated with DDT. Neoplastic tissue formation was the most obvious symptom of environmental pollution in the gonadal tissue of soft shell clams, *Mya arenaria* (Barber, 1996).

The interactions between more than one stressor are discussed by Cairns et al. (1975). They suggested that the effect may be synergistic, additive or antagonistic, and the combined stressors have less effect on the micro-organism than the individual stressor does alone. Also, Duncan and Klaverkamp, (1983) found an increased resistance of Cd in the white sucker pre-exposed to different concentrations of Zn. That was in agreement with our findings of histology measurements. Where polluted + Cd mussels showed some improvements in digestive gland histology and upregulation of *mt10* expression (Chapter 7) over the reference + Cd group. These results were in accordance with an earlier study of Cd chronic exposure followed by heat shock exposure (Veldhuizen-Tsoerkan et al., 1991b). They reported increased synthesis of heat shock proteins after chronic exposure followed by heat shock stress, but not the chronic exposure only. On the other hand, exposed mussels from the polluted site to Cd exacerbated the effect of pollution more than Cd exposure alone, inhibited the phagocytosis ability of haemocytes, more severe pathology in gonads histology, decreased essential metals (Zn and Cu) in some tissues and increased non-essential metals accumulation was observed. Comparing field to laboratory studies reveals the complexity of the effects of the environmental factors which interact with organisms responses and alter them. Furthermore, the presence of mixture of pollutants and their interaction with biotic and abiotic factors in the field make it difficult to interpret the outcome of the field/laboratory animals` response. One of the studies which showed an antagonistic effect of exposure to multiple stressors was the study by Howard and

Hacker (1990). They reported the highest mortality in the grass shrimp, *Palaemonetes pugio* under a combination of temperature, salinity and Cd exposures in the laboratory.

6.5 Conclusion

In our study, we investigated a battery of seven biomarker responses to pollution history (field site) and subsequent exposure to Cd, and measured the deleterious effects of long-term pollution combined with short-term laboratory exposure to Cd. The site effect dominated the responses in this study, where the impacted site showed poor biological measurements compared to the clean site. In general, pre-exposure to pollutants altered the animals' response to Cd exposure in the laboratory. Exposure history affected metal accumulation or distribution in some but not all of the investigated tissues. However, Pb only in gonads is affected by prehistory exposure in the field and not the Ca content in the examined tissues. Pre-exposure history modulated haemocytes immune functions of mussels exposed to Cd in the laboratory. Haemolymph electrolytes, and condition index showed no history exposure or Cd treatment effects, but tissue electrolytes showed some significant disturbance under the effect of site/treatment. Exposure history had an inconsistent effect on the pathology of the digestive gland and male and female gonads. In some cases, exposure history + Cd treatment showed improvement; in others it becomes worse with no additive effect. So, pathological changes depend on tissue type.

Chapter 7

Effect of pollution history and Cd exposure on *mt10* gene expression

Chapter 7

Effect of pollution history and Cd exposure on *mt10* gene expression

Abstract

Aquatic organisms can be exposed naturally to complex mixtures of contaminants in their environment. This "exposure history" will influence their response to contaminant challenges in the future. The effect of exposure history on subsequent responses of gene expression to trace metal exposure is unclear. The marine mussel, *Mytilus edulis*, was collected from reference and polluted sites in South-West England and groups of mussels from each site were exposed to 20 μg l^{-1} $CdCl_2$. The metallothionein (*mt10*) gene expression was estimated after 8 days in the digestive gland and compared to unexposed controls. The 18*S* rRNA gene was chosen as a house-keeping gene. Ct (threshold Cycle) values of rRNA 18*S* gene were not significantly different between samples (ANOVA, $P > 0.05$). *mt10* demonstrated 3 folds induction in the Cd-treated animals from the polluted site compared to all other groups (Kruskal Wallis, $P = 0.01$). We conclude that metal pollution/Cd treatment modulated *mt10* gene expression in *M. edulis*, but pre-exposure history alone has limited effects on this response.

7.1 Introduction

The identification of particular molecular responses, combined with physiological and behavioural changes in the organisms after exposure to pollution is a promising tool for biomonitoring impacted ecosystem (Depledge, 1994). Gene expression is now widely used as a biomarker for environmental contamination and to characterize metal toxicity and responses to one compound or to mixtures of chemicals (Ryan and Hightower, 1996; Halligan and Lunec, 2008). Modification/modulation in gene structure and subsequent expression of the protein may lead to direct biochemical disturbances in cells. For example, microarray analysis was done on *Daphnia magna*

using different cadmium concentrations (10, 50 and 100 μgl^{-1}) for two time points (2 and 4 days) recorded effect on the molecular pathways associated with processes such as digestion, oxygen transport, cuticular metabolism and embryo development (gene arrays, eg., Soetaert et al., 2007). Twenty four genes from mussel (*Mytilus spp*) like *mt10*, *mt20* and *p-53* were analysed after exposure to a Hg/crude North Sea oil mixture (Dondero et al., 2006b), or changes in a few key genes can be used as a biomonitoring tool, where the pattern of changes in the genes can enable comparisons of impacted to reference sites (Neumann and Galvez, 2002). A molecular study on *M. edulis* collected from polluted and unpolluted sites were subjected to aerial exposure and increased temperature (Veldhuizen-Tsoerkan et al., 1991a). The authors found a significant enhancement effect of pollution on the heat-shock protein synthesis after exposure to the pollution plus aerial exposure and increased temperature.

One of the most widely used groups of genes for monitoring metal exposure to mussels in the environment are the genes for metallothioneins. Metallothioneins are considered as a biomarker for specific stressors like metal exposure (Benson et al., 1990). Metallothioneins are low molecular weight metal binding proteins that chaperone metals around the cell, and have a normal role in zinc homeostasis (Hogstrand and Haux, 1996). Two important isoforms are *mt10* and *mt20*, weighing 10 and 20 kDa, respectively (Lemoine et al., 2000). Both mRNA isoforms are expressed in the digestive gland and to a lesser extent in gills of *M. edulis* (Baršyté et al., 1999). MTs are induced mainly by exposure to metals (Soto et al., 1996, Viarengo et al., 2000, Dondero et al., 2006b), although changes in MT expression are also reported during oxidative stress (Roesijadi et al., 1997) and inflammation (Baršyté et al., 1999). Metallothionein proteins are a good monitoring tool of metal pollution in tissues other than measuring metal concentrations in water, sediment and biota with consideration of season, sex, and maturation of the organism (Hylland et al., 1998).

The aims of this chapter were to evaluate *mt10* gene expression under the effect of pollution history with or without Cd treatment in comparison to reference site mussels' gene expression. Also, we aimed to develop a method for the extraction of RNA from field collected mussels.

7.2 Materials and methods

All chemicals were obtained from SIGMA, UK unless otherwise stated.

7.2.1 *mt10* RT-PCR assay

7.2.2 RNA extraction

Digestive gland tissue was used for RNA extraction, and frozen in liquid nitrogen then stored at – 80 °C until required. Total RNA of nine mussels was extracted using QIAGEN RNeasy Mini Kit (Cat # 74104). Tissue (≤ 25 mg) was placed in an Eppendorf tube on crushed ice, containing 600 µl of lysing solution (10 µl 2ME [2-mercaptoethanol] + 1 ml of RLT buffer). A sonication step was introduced into the protocol as part of the method development to confirm full break down of the tissue/cells to improve the quality of extracted RNA. Tissues were sonicated (Misonix, Microson XL, 20 levels) on level 4-5 for 15 sec to break the cells. Then samples were immediately stored on ice while other samples in the same batch were being processed. The tissue lysate were centrifuged for 3 mins at max speed (Micro Centaur, MES, 13000 rpm), and the supernatant containing the RNA was carefully transferred to a new Eppendorf tube. 600 µl of 70% ethanol was added to the supernatant, and mixed. Then 600 µl of the sample, including any precipitation, was added into an RNeasy minicolumn placed in a 2 ml collection tube. Samples were centrifuged for 15 sec at > 8,000 *g*, the eluted flow-through was discarded leaving the RNA attached to the silica gel. The same was done to the rest 600 µl of the sample, using the same tube.

7.2.3 DNA digestion

In order to get a pure RNA sample, DNase digestion protocol was used to digest any DNA contamination in the RNA samples. For DNase treatment of the RNA samples, 350 µl buffer RW1 was pipetted into the RNeasy mini column, and centrifuged for 15 sec at > 8,000 *g* to wash, and flow-through was discarded. 80 µl of DNase (10 µl DNase I stock solution and 70 µl buffer RDD) gently mixed by inverting the tube, was directly added onto the RNeasy silica-gel membrane, and was placed on the bench top (20-30°C) for 15 min for DNA digestion.

Then 350 µl Buffer RW1 was added into the RNeasy mini column to wash the RNA samples attached to the silica gel, and centrifuged for 15 sec at < 8,000 *g*, and the flow-through was discarded. The column was transferred into a new 2 ml collection tube, 500 µl buffer RPE was pipetted onto the column, centrifuged for 15 sec at > 8,000 *g* to wash the column, and the flow-through was discarded. Another 500 µl RPE buffer was added onto the column, centrifuged for 2 min at > 8,000 *g* to wash the column again, and flow-through was discarded, and was centrifuged again at > 8,000 *g* for 1 more min to dry the RNeasy silica-membrane.

The column was transferred into a new 1.5 ml Eppendorf tube, 30 µl of RNase-free water was directly pipetted onto the membrane. The column was left for 1min to sit then centrifuged for 1 min at > 8,000 *g* to elute the RNA. The total RNA concentration was determined by measuring the absorbance of the RNA samples at 260 nm for maximum absorption of nucleic acids as concentration of ng μl^{-1}, 260/280 (RNA/DNA absorbance) ratio was 2.08 – 2.21 and 260/230 (RNA/protein absorbance) was above 1.5 using Nanodrop spectrophotometer (ND-1000) (Table 7.1, Fig. 7.1). RNA elute samples were stored in a freezer at -80°C.

Table 7.1 Total RNA concentration, 260/280 and 260/230 ratio in reference control, reference + Cd, polluted control and polluted + Cd of digestive gland samples

ID	conc. ng μl^{-1}	260/280 ratio	260/230 ratio
Reference control samples			
6c	3141.5	2.1	2.3
7c	885.1	2.2	2.4
16c	524.8	2.2	2
17c	2590.7	2.1	2.3
26c	1403.7	2.2	2.4
27c	2717.3	2.1	2.3
6`c	1123.1	2.2	2.3
7`c	2231.5	2.2	2.3
16`c	1001	2.2	1.7
Reference + Cd samples			
6L	1331.7	2.2	2.3
7L	1380.5	2.2	2.4
16L	1310.2	2.2	2.3
17L	2072.8	2.2	2.3
26L	573.8	2.2	1.4
27L	2226.6	2.2	2.3
6`L	1122.6	2.2	2.3
7`L	563.9	2.3	2
16`L	822.2	2.2	2.3
Polluted control samples			
6h	1053.8	2.2	2.3
7h	116.5	2.2	2.2
16h	1798.1	2.2	2
17h	416.6	2.2	2.3
26h	1660.8	2.2	2.3
27h	1587.6	2.2	2.3
6`h	1939.3	2.2	2.4
7`h	2898.7	2.1	2.3
16`h	1485.7	2.2	2.3
Polluted + Cd samples			
6i	1493.9	2.2	2.3
7i	796	2.2	2.3
16`i	161.7	2.2	1.7
17i	2112.1	2.2	2.3
26i	2186.1	2.2	1.9
27i	2337.5	2.2	2.3
6`i	2565.5	2.2	2.2
7`i	3046	2.1	2.2
16i	1058.4	2.2	2.3

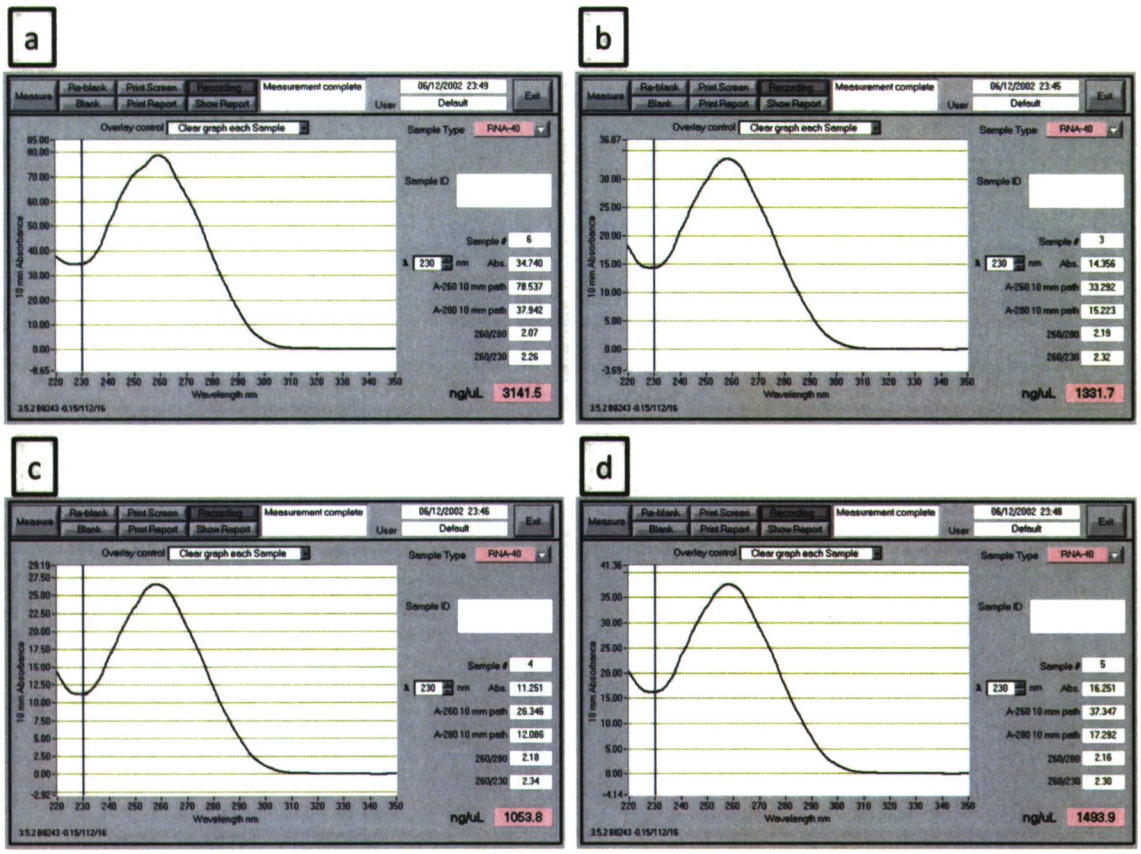

Fig. 7.1 Confirming the quality of RNA extraction from digestive gland samples of *M. edulis* measured by the NanoDrop spectrophotometer. (a) reference control, (b) reference + Cd, (c) polluted control, and (d) polluted + Cd. A clean RNA extraction should yield a distinct peak at 260 nm and with no noise on the spectrum (a smooth line).

7.2.4 Reverse transcriptase (cDNA)

To convert the total RNA samples to cDNA material, the reverse transcriptase method was used. cDNA was synthesised using 1-5 μg of total RNA and was incubated with reverse transcriptase (M-MLV Reverse Transcriptase, SIGMA, Cat # M1302). dNTPs (Deoxynucleotide triphosphate sodium) mix plus random hexamers (1.6 ml of molecular water was added to the 1 unit vial for final concentration 1-5 μM) were added to each sample of 2 μl mRNA [1000/sample conc. (ng/μl) = volume in μl] as Master Mix A (Table 7.2). Then q.s. Nuclease-free water was added to complete 10 μl.

Table 7.2 Preparation of cDNA Master Mix A and Master Mix B

Chemical name	Volume	Master Mix type
10 mM dNTPs mix	1 μl	A
Random hexamers	1 μl	
Total volume	2 μl	
10X M-MLV Reverse Transcriptase Buffer	2 μl	B
M-MLV Reverse Transcriptase	1 μl	
Molecular Water	7 μl	
Total volume	10 μl	

Note. M-MLV, Moloney Murine Leukemia Virus.

The mixture was briefly centrifuged (e.g., 200 *g*) for 5 sec to gently collect all components to the bottom of the tube and to prevent it from sticking to the wall of the Eppendorf. The mixture was incubated (Gene Amp PCR system 9700) at 70 °C for 10 min. Then the tubes were immediately removed and placed on ice for 5 min. 10 μl of Master Mix B (Table 7.2) was added to each previously incubated sample to give a final volume of 20 μl. The reaction was incubated at room temperature for 10 minutes to ensure elongation of random primers before the higher reverse transcriptase temperature. The reaction was incubated at 37 °C for 50 minutes, 94 °C for 5 minutes to denature the M-MLV reverse transcriptase, and then preserved at 4 °C for a month, or -20 °C for longer.

Primers of *mt10* were designed using the mRNA sequence of the *mt10* gene which was obtained from a Primer Blast in the NCBI (National Centre for Biotechnology Information) GenBank: (Accession No. AJ005453.1). The criteria for primers included the GC ratio, temperature and product length was: 65 and 59.09 %; 60.25 and 59.86 °C respectively; and using primers of 166 pb for both sense and antisense. Primers for the 18*S* house-keeping gene were designated using rRNA gene sequence which was obtained from Primer Blast in GenBank: (Accession No. L24489). GC ratio, temperature and product length were: 65 and 65 %; 60.11 and 60.39 °C; and 182 pb for sense and antisense, respectively. The RT-PCR analysis included *mt10* using CybrGreen PCR Master Mix (CybrGreen JumpStart Taq ReadyMix, Sigma, Cat # S 4438). *mt10* specific primer pair was sense: 5`- TGCAGCGGTGACGGTTGTCG -3`; *mt10* antisense: 5`- ACTTGCAGGAACAGCCAGGTGC -3`. Data were normalized against the expression of ribosomal rRNA 18*S* target, using the following primer pair 5`- GCGATCCGCCGGAGTTGCTT -3`; 18*S* antisense: 5` TGTCCGGGCCGGGTGAGTTT -3`.

The method development and optimisation took several months and many primers were used to get the best specific PCR product for cDNA of *mt10*. These are shown in Table 7.3.

Table 7.3 List of primers (sense and antisense) sequences used for *mt10* and 18*S* RT-PCR analysis and their characteristics

Oligo name	Sequence (5` > 3`)	No. base pairs	Tm (°C)	GC content %	Reference
mt10					
Sense	GGGCGCCGACTGTAAATGTTC	21	61.8	57.1	Dondero et al. (2006)
Antisense	CACGTTGAAGGYCCTGTACACC	22	63.1	56.8	
Sense	CGACGTCGCCACTGCCAACA	20	63.5	65	Primer Blast, GenBank
Antisense	GTGAACGTCCTTGTCGGTCCACG	23	66	60.9	
Sense	**TGCAGCGGTGACGGTTGTCG**	**20**	**63.5**	**65**	**Primer Blast, GenBank**
Antisense	**ACTTGCAGGAACAGCCAGGTGC**	**22**	**64**	**59.1**	
18*S*					
Sense	TCGATGGTACGTGATATGCC	20	57.3	50	Dondero et al. (2006)
Antisense	CGTTTCTCATGCTCCCTCTC	20	59.4	55	
Sense	**GCGATCCGCCGGAGTTGCTT**	**20**	**63.5**	**65**	**Primer Blast, GenBank**
Antisense	**TGTCCGGGCCGGGTGAGTTT**	**20**	**63.5**	**65**	

Note. The highlighted primers are the ones that worked the best for *mt10* and 18*S* from *Mytilus* tissue. Note. Tm, temperature.

7.2.4 Gel electrophoresis for PCR product

In order to check the purity and molecular weight characteristics of the DNA of PCR product, agarose gel electrophoresis was run. The DNA was produced by the ordinary PCR machine and was separated by gel electrophoresis in order to confirm the size, location and quality of the PCR specific product for specific primers of *mt10* cDNA. The electrophoresis tank was prepared using a standard method (Sambrook et al., 1989). Gel was prepared by mixing 70 ml of 1x TAE (Tris Acitate EDTA) with 0.7 g agarose, and was micro waved for 2 mins on medium/high power. Then, 2.5 µl of 500 mg/l Ethidium Bromide solution was added to the gel mixture and dissolved in the warm gel. Finally, 2.5 µl of the Orange G dye (1/10 sample volume) was added to each sample. For calibrating the gel, 25 µl of a 1 kb ladder was prepared as follows; 21.5 µl of molecular water, 2.5 µl of Orange G dye and 1µl of ladder (100 bp, Promega). The gel was left to warm, then was poured into an electrophoresis tank (Pharmacia Biotech, GNA 100, 8x10.5 cm) and was left to solidify. Then the electrophoresis tank was filled with 1x TAE. 25 µl of each sample was loaded into the wells starting with the ladder. The electrophoresis tank then was covered and connected to the power on 40 volts for 1 hour. DNA was visualized and photographs were taken of the gel using UVItec Limited, England.

7.2.5 RT-PCR for *mt10*

RT-PCR experiments were run to compare semi-quantitatively *mt10* gene expression in experimental groups relative to the reference control group. RT-PCR was run using comparative experiment to compare *mt10* gene expression in the mussels of the control reference site to the *mt10* gene expression of reference + Cd, control polluted and

polluted + Cd. The CybrGreen PCR Master Mix was prepared (for one reaction) as shown in Table 7.4. Forty-eight well plates were used for RT-PCR reactions, in an RT-PCR machine (Applied Biosystems, 271000885). Negative controls and samples for target and house-keeping gene were done in triplicate. Each sample was run three times by RT-PCR protocol to confirm the results from 9 mussels/treatment.

Table 7.4 CybrGreen PCR Master Mix preparation for thermocycling

Chemical name	Volume
2x JumpStart *Taq* ReadyMix	12.5 µl
Reference Dye 1x	0.25 µl
Forward Primer (10 µM)	0.5 µl
Reverse Primer (10 µM)	0.5 µl
Template cDNA	2.0 µl
Molecular Water	9.5 µl
Total Volume	25 µl

The thermal protocol for PCR reactions were as the following: 3 min incubation at 95 °C, followed by 40 reaction cycles: 15 s at 95 °C, 30 s at 55 °C, 20 s at 72 °C where the fluorescent amplification signal was read (Dondero et al., 2006a). A melting curve of PCR product was adjusted between 60 - 95 °C. Each experiment of RT-PCR was done by triplicates for each target for a total of 36 samples for all groups. The amount of *mt10* expression data was analysed based on the differences between the reference control group and other treatments by using a comparative Ct experiment, where Ct is threshold cycle by using the following formula according to Livak and Schmittgen, (2001):

$\Delta Ct_{(sample)} = Ct_{(mt)} - Ct_{(18S)}$

$\Delta\Delta Ct_{(sample)} = \text{sample } \Delta Ct_{(mt)} - \text{reference control } \Delta Ct_{(mt)}$

$\text{Amount of target (RQ)} = 2^{-\Delta\Delta Ct\,(sample)}$

7.3Results

7.3.1 Quantitative real time PCR analysis for *mt10*

An expression of *mt10* transcripts was estimated in the digestive gland of mussels on day 8. Ct values of 18*S* as a house-keeping gene were not significant between groups (ANOVA, $P > 0.05$). The highest values for the *mt10* target were recorded at the Polluted + 20 $\mu g\ l^{-1}$ Cd treatment, and to a lesser extent in the 20 $\mu g\ l^{-1}$ Cd alone group. The addition of 20 μgl^{-1} Cd significantly upregulated the expression levels of *mt10* in the polluted + Cd-treated mussels (Kruskal Wallis, $P = 0.01$) when compared to the other groups. The polluted + Cd group was higher with 3.01, 4.7 and 2.3 fold induction of *mt10* compared with the reference control, the polluted control and reference + 20 μgl^{-1} Cd, respectively (Figs. 7.2 and 7.3, Table 7.5).

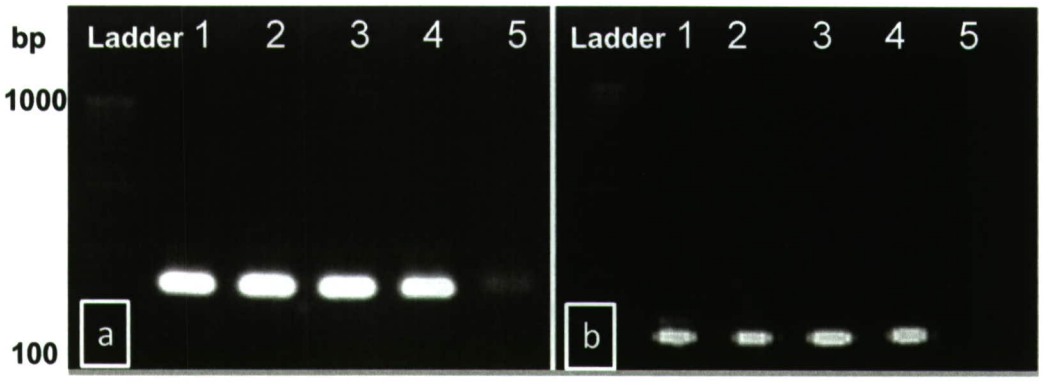

Fig. 7.2 Electrophoretic photographs of PCR products, (a) 18*S* gene and (b) *mt10* gene, in reference control (lane 1), polluted control (lane 2), reference + Cd (lane 3), polluted + Cd (lane 4), and blank (lane 5) in 1.5 % agarose gel and 1 kb ladder.

Table 7.5 Output of RT-PCR expression analysis of mussels' *mt10* and 18*S* genes

Site	Treatment	Target ID	Target Ct	S.E.M.	Normalized expression
Reference	Control	18*S*	7.3	0.9	
		mt10	23.4	0.7	
	+ Cd	18*S*	7.9	0.7	
		mt10	22.6	0.7	1.3
Polluted	Control	18*S*	8.2	0.9	
		mt10	23.3	1.1	0.6
	+ Cd	18*S*	8	0.8	
		mt10	22.5	1.1	3.0

Data are mean threshold cycle (Ct), the normalized expression values are relative to the reference control. Standard error means (S.E.M.) obtained from the amplification of *mt10* gene and an 18*S* ribosomal target used for normalization of expression data. Data represent the mean of three independent triplicates analysis of each mussel, $n = 9$ mussels per treatment.

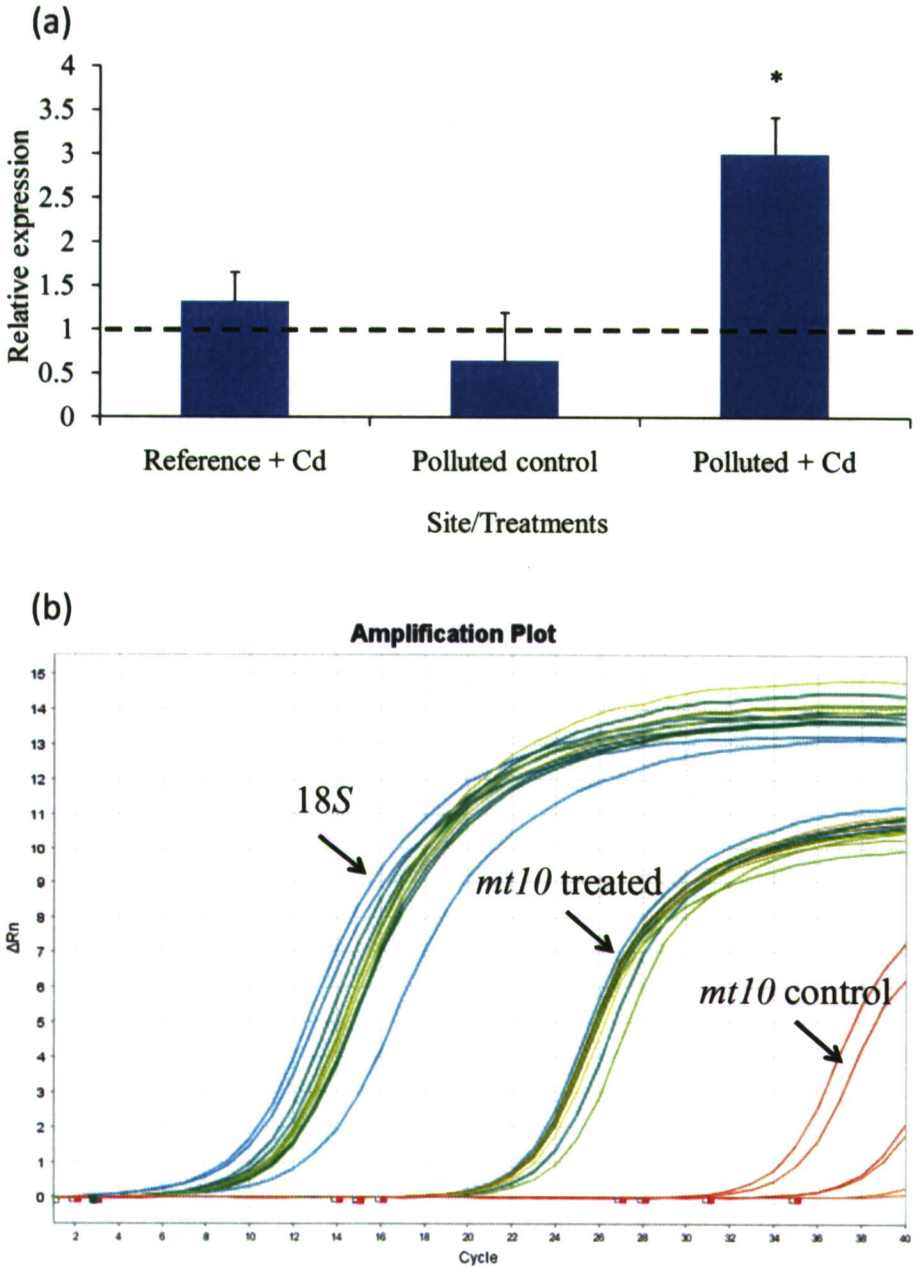

Fig. 7.3 Effects of Cd on *mt10* gene expression levels (a) and amplification plot of *mt10* and 18*S* Ct values (b). Data (mean ± SEM, n = 9) are expressed as relative respect (dashed line) to reference control mussels showing upregulation of gene expression in reference + Cd, and polluted + 20 μg l^{-1} $CdCl_2$. * indicates a significant difference from reference + Cd and polluted control groups, $P = 0.01$, Kruskal Wallis.

7.4 Discussion

7.4.1 Method development for metallthionein genes in field-collected mussels.

RNA was extracted from digestive gland cells using the sonication method on ice to ensure breakdown of every cell in the tissue and of the same time keep the total RNA free from damage. The DNA digestion step was applied even with the high performance of the mini column method of RNA extraction to increase the quality of our samples. Then extracted RNA samples were measured using Nanodrop (at 260, 280 and 230 nm) to confirm the purity of our RNA samples from DNA or protein contamination. The method of using a spectrophotometer to confirm the quality and purity of RNA is widely used by previous authors (Dondero et al., 2006 a and b; Fasulo et al., 2008). Primers were applied first according to previous literatures. When the PCR product were checked and visualized by gel electrophoresis and found to be weak, others were designed using Primer Blast depending on RNA sequence of 18*S* and *mt10* obtained from GenBank data. PCR products were confirmed by its size and purity using gel electrophoresis. Q-PCR products were usually evaluated by agarose gel electrophoresis as explained by Dondero et al. (2005) and Fasulo et al. (2008). The melting temperature of the PCR product of *mt10* was checked online using Biomath Tm calculations for Oligos (Promega) and was convenient with the amplified PCR product obtained from RT-PCR machine. Precautions should be taken to differentiate between the melting and working temperatures of the primers.

7.5.2 Effect of Cd exposure on regulation of *mt10* gene expression

The *mt10* gene expression was estimated in the digestive gland on day 8 in the control from the reference site, the polluted site, reference + 20 $\mu g\ l^{-1}$ Cd treated mussels, and

polluted + 20 μg l^{-1} Cd mussels (Figs. 7.2 and 7.3, Table 7.5). There was some expression in the reference control site. Similar to many other genes in whole organisms, *mt10* also has a detectable background of gene expression. This is not surprising given the normal biological role of metallotheineins in zinc metabolism (Hogstrand and Haux, 1996), and inevitably, even animals from "pristine" ecosystems may have background levels of metals (e.g., a few ng/g_of each metal) that together might require a metallotheinein chaperone. Background expression of *mt10* has been previously reported in unexposed controls in experiments using *M. edulis* (Baršyté et al., 1999). Dondero et al. (2005) also argue that *mt10* can be normally transcribed either in the presence or absence of metal loads. Other chemicals are also known to induce MT expression in animals, especially those associated with oxidative stress (e.g., hydrogen peroxide, Gasch et al., 2000).

Animals from the reference site + Cd showed *mt10* expression levels that were marginally higher than animals from the polluted control site (not statistically significant, Fig. 7.3a). This is at least consistent with higher tissue Cd levels in animals from the treated mussels with Cd from the reference site, but the increase in gene expression was much higher when polluted site animals were exposed to Cd in the laboratory (Fig. 7.3a). In Cd exposed animals from the polluted site (polluted + 20 μg l^{-1} Cd), a statistically significant (about 3-fold) induction of *mt10* was found in the digestive gland (Fig. 7.3a and table 7.5). The increase in expression was not surprising. Previous reports show 200 μg l^{-1} Cd can cause upregulation of MT genes in gills of *M. edulis* (Soazing and Mark, 2003). There may also be an upper limit to the induction. Dondero et al. (2005) recorded a maximal induction of *mt10* (24 fold) by treatment with 200 μg l^{-1} Cd over 6 days in *M. galloprovencialis*.

Animals from the reference site were also exposed to Cd in the laboratory (reference + 20 μg l^{-1} Cd treatment). These animals also showed some induction of *mt10*, and the trend was higher than reference animals alone (no Cd exposure in the laboratory), but lower than the same Cd exposure done on the mussels from the polluted ecosystem (Fig. 7.3). This implies that the previous exposure history and/or health of the animals will alter *mt10* expression, and that animals from polluted sites can show relatively more *mt10* expression. Some of this may simply be stoichiometry of the metallothionein protein which has four metal binding domains (Winge and Miklossy, 1982). If the tissue has more metal, then it may simply need more metallotheionein to chaperone it around the cell. It may also be related to Cd dynamics in the tissue and control of free metal ion concentrations. Blackmore and Wang (2002) mentioned that Cd pre-exposure increased the assimilation efficiency (AE) for Cd, as well as increasing the overall Cd tissue burden in *Perna viridis*. One possible reason for increased *mt10* expression is therefore the rate of Cd turnover, with *mt10* trapping newly acquired Cd as it enters the digestive gland, and consequently maintaining a low level of intracellular free Cd.

However with MTs responding to several trace elements, the dynamics can be complicated. For example, Soto et al. (1996) found that Cu and Zn also induce mettallothiones in tissue of the marine winkle, *Littorina littorea*. Clearly, some metals share similar uptake pathways due to their affinity to bind to the same/similar ligands (Cd v Ca, Zn v Cu, etc., Amiard et al., 2006). So, MT induction by one metal can affect the regulation of the other trace metals (Wang and Dei, 1999). Like inorganic Hg toxicity tolerance in gills of *M. edulis* after pre-exposure to Cd, Cu or Zn where the later induced MTs-like proteins (Roesijadi and Fellingham, 1987). Clearly, it is a combination of the Cd concentration in the tissue, its rate of turnover and the dynamics of other trace metals that contribute to the overall expression of *mt10*. In our study,

exposure to Cd in the laboratory challenges the metal regulation systems of the test organisms, and reveals the ecosystem site differences between reference and polluted locations.

7.5.3 *mt10* from *Mytilus* as a potential biomarker

In our experiment we found significant changes in *mt10* gene expression in the digestive gland of the marine mussels *Mytilus edulis* under the effect of Cd exposure after pre-exposure history of the polluted area. However, to compare clean site mussels to polluted site *mt10* gene expression, there was no significant regulation found between them. But adding more stressors, such as Cd treatment in our experiment made the significant difference in the expression. So, using a molecular approach in biomonitoring the environment is a sensitive tool but under specific conditions. Because metallothoioneins have dual action in homeostasis of essential metals (Hogstrand and Haux, 1996) and detoxification of non-essential metals (Roesijadi et al., 1982). It is difficult to predict what role was responsible for up/down regulation of their gene expression. Other reasons may be attributed to internal or external factors affecting MTs genes expression in the organisms. An example of unreliability of this biomarker was confirmed by Geffard et al. (2005) who mentioned that MT concentrations in gills of *Mytilus edulis* was not reliably reflecting metal contamination.

7.5 Conclusion

In conclusion, a method was developed to measure *mt10* expression in field-collected mussels. The results of this study show that there is a significant increase in *mt10* expression in the digestive gland of *M. edulis* on day 8 after exposure to Cd in animals collected from a polluted site, and this response was different from animals exposed to the same Cd concentration, but collected from a pristine ecosystem. This suggests that previous exposure history is important in *mt10* expression in *M. edulis*. Our results suggest that *mt10* alone cannot detect differences between ecosystems, but further studies using different concentrations of Cd exposure and different time points are needed to clarify the *mt10* pattern of expression in the digestive gland.

Chapter 8

General discussion

General discussion

Our work showed many adverse effects of trace metals represented by Hg and Cd on the tissues and cells of marine mussel *Mytilus edulis*. The physiological effect on haemolymph electrolytes proved that the animals have the efficiency to regulate and control electrolyte balance of the blood cells (Chapters 3 - 6). Immune modulation effects were induced by sublethal concentrations of Hg and Cd (Chapters 3 - 6), where treatment initiates/inhibits immune functions, but not dramatically. Gene expression of *mt10* was a confirmation that the animal responded to the stress successfully and it explained why the animal was tolerant of the experimental concentrations, and why these were not lethal and caused animal death (Chapter 7). Histopathology reactions such as inflammation and necrosis are a good snapshot of what the cells and tissues can do to react to metal accumulation (Chapters 3-6). Toxicity and death due to exposure to metals such as Hg high concentration (500 $\mu g\ l^{-1}$), could perhaps result from the fail use of animal defences to cope with overload/spillover of toxic form of metals (Chapter 3).

8.1 Target Organs for Hg and Cd in *Mytilus edulis*

Hg and Cd are representative toxic metals which are biologically non-essential. These metals showed accumulation in tissues and haemolymph of *M. edulis*. Both exposures were performed using the chloride salts of the metals, and this is highly relevant to the environmental risk assessment as these chemical forms are mostly discharged in industrial effluents. The expected pattern of metal accumulation is that the target organs reflect the route of exposure, with the gill showing contamination from aqueous exposures. In this study, gills from all the experiments showed metal accumulation as expected, but it was interesting that the pattern for internalisation of Hg was different to Cd. For example, Hg was mainly accumulated in the gills (Chapter 3) while Cd

accumulated in the digestive gland (Chapter 4) of *M. edulis*. Mussels have an open circulation and so there is unlikely to be a cardiovascular aspect to the differences between Hg and Cd. However there may be a chemical explanation. For example, the Cd binds much more avidly to thiol groups on proteins and organic residues on lipids (e.g., apparent log K' with lipid, Hg^{2+}, 4.49; Cd^{2+}, 6.89; Ochoa-Loza et al., 2001). Hence, Cd may have been firmly trapped in more fatty tissue like the digestive gland. To confirm this idea it would be useful to measure the metal binding affinity and capacity of the different tissues, as Hollis et al. (1997) did for fish gill.

This metal binding affinity may also offer an explanation for the different EC_{50} of each metal; 14.7 and 110 mg l^{-1} for Cd and Hg respectively in *M. edulis* (Sauvé et al., 2002). Both Hg and Cd concentration in tissues followed the seawater concentrations, so there was no deviation from the concept of concentration-effect in terms of metal accumulation, as with many other metals (Odzak et al., 1994). However, there may be differences in bioavailability. The lower concentrations of metal exposure had more effect on the mussels than the higher concentrations (Chapters 3 and 4). Some of this is easily explained by closure of the shell at higher doses, but that may not be full the explanation in terms of biological effects. Trace metals like Cu, Cd, Hg, Zn and Pb have a relatively high affinity for SH groups of proteins, and Viarengo et al. (1993) argue that enzymes such as lipid peroxidases will be damaged leading to an increase of oxyradical production and toxicity. Of course, such arguments require free metal ions to be available to SH groups, but in reality responses are also complicated by the induction of metal binding proteins in cells. Cd caused upregulation of *mt10* (Chapter 7). It may also be that the same or similar metal binding proteins are used for both Hg and Cd in *M. edulis*. Roesijadi and Hall (1981) reported that the amino acid composition of mercury-binding protein was similar to that of cadmium-binding protein of *M. edulis* mussels' gills.

The distribution of Hg and Cd in tissues of mussels other than the main/initial target organs was found to be the same. In our study, digestive gland and adductor muscle were a lesser accumulative organs for Hg accumulation, and gills and adductor muscle for Cd accumulation. Clearly, haemolymph was not a target compartment for any of them and it showed opposite response to tissue accumulation as observed by (Hemelraad et al., 1990). In Cd exposure following chronic field pollution experiment, gonads recorded lesser accumulation of Cd in the reference, the treated mussels (Chapter 6) and the polluted mussels of the field work (Chapter 5).

8.2 Effects of Hg and Cd on the immune responses of *Mytilus edulis*

Both Hg and Cd had similar effects on the immune response of the mussels. For example, the neutral red uptake and phagocytosis responses showed transient alteration/modulation under exposure. These findings are similar to other trace metals like Cu (Pipe et al., 1999), Sn and Zn (Fisher et al., 2000). Cheng and Sullvian (1984) reported variable patterns of phagocytosis responses to Co, Cr, Cu, Fe, Hg and Cd in the American oyster, *Crassostrea virginica* haemocytes, where no effect, inhibition / stimulation of phagocytosis ability was dependent on metal species and its concentration. During the current study, inflammatory reactions were approved in the examined tissues. Cheng (1986) discussed the idea of molluscs that during inflammation, over synthesis of lysosomal enzymes is released in the haemolymph under the effect of challenge with foreign substances. Thus, in our study, mussels are giving similar reaction responses to Hg and Cd molecules as non-self molecules. As mentioned by Lowe et al. (2000) when organisms are under pollution condition, lysosomal membrane stability will be affected and leakage of lysosomal enzymes can

damage the cells. It seems that may happen with digestive gland lysosomes which cause damage to the digestive tubules and that was shown as tissue pathology.

8.3 Metal pathologies in *Mytilus edulis*

Histopathological biomarkers were defined and recommended by Hinton et al. (1992). Tissue pathology was found to be slightly different and some symptoms which were associated only with Hg exposure, but the majority of symptoms are the same, for example, the inflammatory reactions, cell necrosis, and disorganization of cellular structures. Similar patterns of tissue pathology have been recorded with other trace metals. A mixture of Cu and diesel oil caused inflammation and necrosis in the tissues examined, gills, gonads and digestive gland of bivalve molluscs (Auffret, 1988; Hinton et al., 1992). In the digestive gland, Hg effects on digestive tubules include necrosis and inflammation plus vacuolization in the epithelium (Chapter 3). Similar to Hg, Cd effects in digestive gland were found to be the same except those of vacuolization. Inflammation and necrosis are expected to be symptoms as a host reaction occurs during metal toxicity (Da Ros et al., 1995). The gill filaments were the affected sites for both Hg and Cd. Erosion of cilia and necrosis of epithelium as toxic effect of metal exposure was shown in many aquatic animals (e.g., Fasulo et al., 2008). These pathologies will definitely impair the gill respiratory and feeding functions. More laboratory work is needed on feeding rate and respiration to support that conclusion experimentally. Loss of muscle architecture in addition to inflammation was the main pathologies of adductor muscle. This was clearly observed in animal with fully opened valves of mussels before death under the high concentration of Hg. This means that adductor muscle lost its capacity to close the two valves together, thus the mussels lost their first line of defence to exposure. Long opened valves, absence of mechanical stimulation were not observed

in Cd exposure, because the exposure concentration did not reach the lethal concentrations, and there was no mortality recorded during Cd experiments. In conclusion, toxicity of metals depends on many factors like target organ, concentration, type of metal, and species sensitivity and many others.

8.4 Responses of *Mytilus edulis* as environmental biomarkers

The many reasons why invertebrate animal was chosen as a model are discussed in the following paragraph. Because invertebrates are account for 95 % of all known species in the animal kingdom and are extremely important as a part of ecosystem structure and function, and their wide geographical and ecological distribution (Oehlmann and Schulte-Oehlmann, 2003), biomarker measurements recommended to be done on invertebrates. Furthermore, there are numerous populations and sampling them will not affect their or other population dynamics and they do not need a license to work on them (Depledge and Fossi, 1994). Lagadic et al. (1994) added they are suitable for experimental manipulation because of their high growth rate, short generation time, small size and reasonably maintenance space. They can be used in diagnostic and predictive biomarkers; their high growth rate and short generation time allow rapid assessment of the ecological restoration as a primary indicator.

Molluscs, especially bivalves have a good reputation in biomonitorig programs, thus our choice was made depending on the following reasons. As molluscs possess an open circulatory system, they are in continuous exposure to fluctuations of environmental factors and pollutants (Cheng, 1981). Because of their lifestyle as stationary, filter feeders, low metabolism and inhabiting the benthic environment with no escape, bivalves can be in continuous contact with pollutants in a fixed

location. In contrast, in highly mobile organisms such as fish, it is difficult to predict the kind of pollution they suffered from. Besides, mussels gave good examples of comparing data base in metal concentration of Mussel Watch Program worldwide (Cantillo, 1988). That can be useful in data comparison of reference and polluted areas all over the world. As a sentinel species, bivalves are widely used in evaluation and collection of data about pollution in marine and estuarine ecosystems (Goldberg, 1989).

8.5 Exposure and sensitivity of end points

Choosing the right organs as a target organ for the biological end point is an important aspect in establishing a biomarker. Measuring the right end point in the wrong tissue can lead to no results at best, and misinterpretation of data. The gills are an obvious target because of their role in food filtration, respiration and their close contact with the external environment (David et al., 2008a). The digestive gland is also a good example as an internal target organ. It is the site of accumulation for many metals (essential and non-essential) (Domouhtsidou and Dimitriadis, 2000) and contains many enzymes including digestive enzymes (Johnson et al., 1996) that could lend themselves as biomarkers. The digestive gland is also an important detoxifying organ (Livingstone et al., 1992; Canesi et al., 1998).

Neutral red uptake recorded sensitive changes in single metal exposure (Hg and Cd, Chapters 3 and 4), in field assessment (Chapter 5) and assessing Cd exposure effect on mussels from pre-exposed naturally to pollutants (Chapter 6). Neutral Red retention method as an immunoassay, which measures the alterations in lysosomal compartments, is a viable means to identify pollution impacts on biota in situ (Galloway et al., 2002). The lysosomal membrane stability as measured in haemocytes of mussels is considered

as a non-destructive mean of assessing metal pollution (Nicholson, 2003). Also, Regoli (1992) confirmed that measuring lysosomal responses in digestive gland of *M. galloprovincialis* mussels is a sensitive index for stress result from chronic heavy metal pollution. Thus, neutral red retention assay is recommended by us as a good biomarker of heavy metal (Hg and Cd) exposure, but more is needed to define external seasonal effects.

Histological analysis and quantification has been suggested as a good biomonitoring tool in environmental risk assessment (Auffret, 1988; Hinton et al., 1992) and to assess chronically exposed environments (David et al., 2008b). In a review by Au (2004) histo-cytopathological biomarkers are easy to determine and histopathological lesions can serve as the primary indicator of pollutant exposure. The histological responses in this study (Chapters 3 - 6) were very detailed and often detected changes when there was little or no effect on immune parameters. Histopathology may therefore be a much better monitoring tool for inflammation events. Nonetheless, the neutral red retention assay has attracted many researchers and the destabilization of lysosomal membrane permeability is a fundamental aspect of cellular toxicity (Moore, 1990; Nicholson, 2003).

Haemocyte counts were found to be an insensitive biomarker (Chapters 3, 4 and 6). However, it is useful to bench mark the haematology of the animal so that the toxic responses can be understood. However, this haematology may be specific to the time and group of animals being studied, as it can fluctuate with seasonal change (Pipe et al., 1995b), as well as with nutritional status (Chapters 3 and 4, not fed) and study site (Chapter 6). However, on the other hand, many researchers recorded that haemocytes increased in number under the effect of exposure to contaminants or stress (Kayama et al., 1995 and Pipe et al., 1999).

Body size of animals is also a confounding factor when measuring physiological responses. It is therefore useful to record the morphometrics of the animals. However, if a narrow range of sizes have been collected from the field, it possible to correlate shell length or body weight to metal concentration (e.g., Chapter 5), but this can be mislead if the ranges of shell sizes are not large enough. To overcome this problem, sampling a wide size range is the solution (Brown and Luoma, 1995). In our study, mussels had similar lengths, and while this was done to reduce sample variability, it also prevented the identification of mussel size and age effects on the metal accumulation or immunological responses.

Metal exposure has a negligible effect on glucose levels in haemolymph of mussels. Lagadic et al. (1994) also argued that haemolymph glucose concentrations in invertebrate were largely insensitive to pollution, and critically affected by season and nutritional status. Also, haemolymph electrolytes were not sensitive enough to give a clear picture of the effect of exposure, because *Mytilus edulis* is an osmoconformer. Shumway (1977) argues that haemolymph Na^{+}, Mg^{2+}, Ca^{2+} concentrations followed the concentrations of the external medium in *M. edulis* (as long as the shell remained open). So, it is perhaps no surprise that the haemolymph electrolytes simply matched the surrounding seawater.

Here we have chosen biomarkers that are promising, and relate to immunity and inflammation. This is only one body system/effect and should not be considered alone. A good environmental monitoring programme will use a suite of biomarkers (Handy et al., 2002) and so the ones we select here should be incorporated with others. For example, detoxification enzymes like mixed function oxidase (MFO) and glutathione-*S*-tranferase (GST) are the more likely to be used as a direct evidence of xenobiotic exposure, and acetylcholinestrase (AChE) and non-specific cholinesterase (ChE)

activities are potential tools in assessing the effects of organophosphate and carbamate pesticides (Lagadic et al., 1994). Glycogen and enzymes involved in carbohydrate metabolism are powerful markers for the bioenergetic effects of pollution (Lagadic, 1994). The adenylate energy charge (AEC) is another example of metabolic state indicator of the cells (Atkinson, 1977). Scope for growth (SFG) is a good quantitative parameter in measuring the energy reserved for growth and reproduction as a pollution impact in *M. edulis* (Widdows et al., 2002). Clearly, a combination of approaches will yield the best understanding of the pollution problem, a "weight of evidence" is crucial for the regulatory use of biomarkers in legislation (Handy et al., 2003).

8.6 Molecular methods in environmental monitoring

Because of the important role of genes in organisms' biological functions, gene expression as a defensive and adaptive mechanism of the animal under the effect of natural pollution and experimental exposure was one of our interests (Chapter 7). Gene expression has been successfully used to assess the stress of environmental changes and add another feature to the genomic response as its specialized induction/inhibition for specific condition (Gasch et al., 2000). DNA microarray is now used in variety of fields including ecotoxicology, predicting genes biological functions, biochemical pathways, drug response and target and toxicological characterestics of biomarkers (Neumann and Galves, 2002; Dondero et al., 2006b). Microarray expression gives a clear snapshot of the gene expression response at a specific time in a specific tissue (Neumann and Galves, 2002).

Real time quantitative PCR is considered the most sensitive and reliable for detection of gene expression levels especially with low amount of mRNAs (Orlando et al., 2005). Furthermore, the type of pollutant can successfully determined by using gene

expression profiles as reported by Dondero et al. (2006b). RT-PCR data from our experiment (Chapter 7) showed uregulation of *mt10* as an effect of Cd exposure after chronic field exposure. Our results confirm that Cd exposure did regulate gene expression in chronically exposed mussels and only Cd exposed mussels. However, some baseline expressions were recorded in the reference control mussels as a result of season effect and as a biological function of *mt10* for essential metal homeostasis. This was in consistence with previous reports (Dondero et al., 2006b). Some precautions and limitations in interpreting data outcome from gene expression should be taken. In Lecoeur et al. (2004) Cd induced MTs in the bivalve *Dreissena polymorpha* soft body but not Cu, and explained that as MTs are tissue specific and can be lost in dosing MT in total soft body. In contrast, Dondero et al. (2006a) found that organic aromatic chemicals did not induce MTs genes in *Mytilus spp* and these represent a robust indicator of trace metal contamination and exposure. As previously mentioned, MTs gene expression can be used to identify the type of pollutant (metal) in the environment. MT analysis in mussels was recommended in the framework of the Mediterranean Action Plan as a biomarker by UNEP/RAMOGE (1999). Neumann and Galves (2002) explained that nutritional status can severely affect gene expression and this can be used to detect the difference in pattern between polluted and clean site animals. In conclusion, there appears to be lots of contradiction on the effect of heavy metal on MTs expression. This can partly be explained by the interference of confounding factors: external environmental factors and internal biological factors and the dual functions of MTs as metal homeostasis and detoxification.

8.7 Revisiting the hypothesis

The original hypothesis focused on exposure/effect mechanisms, and where the mussels were exposed to heavy metals, there would be adverse effects on the animal's biological functions. Thus, we expected to observe metals accumulation, poor immunity, and tissue pathology, as well as disturbance in tissue and haemolymph electrolytes. Most of these elements hypothesis of these supported by our data, although other aspects need revision.

So, a revised hypothesis including the new findings in our current work (Fig. 8.1) was put forward. Environmental pollutants can lead to many changes in the affected organisms. When the pollutant has the chance to enter and pass through the organism's first line of defence, it binds and interacts with different target tissues, depending on the affinity we explained earlier. These tissue /chemical interactions result in changes in the metabolism of the organism tissue and consequently affect/impair their functions. These impairments can be altered behaviour, cause immune dysfunction, and cellular electrolyte disturbances, and alteration in gene expression, tissue pathology such as necrosis or foci which may result in unsuccessful reproduction. Sometimes death is the end step when the organism cannot tolerate chemical toxicity. Otherwise, the animal tried to avoid and deal with most of the adverse effects by haemocyte infiltration to the affected organs to phagocytose the foreign molecules (Chapters 3 – 6), increasing metallothionien production (Chapter 7) to bind and excrete these toxicants outside their bodies. In addition, they controlled their tissue and haemolymph electrolytes by opening their shells to follow the external medium (Chapters 3 – 6). Thus, integrity of assays as a battery of biomarkers is a reliable tool in diagnosis of organism's health and predicting pollutants adverse effects on the organisms' biological functions.

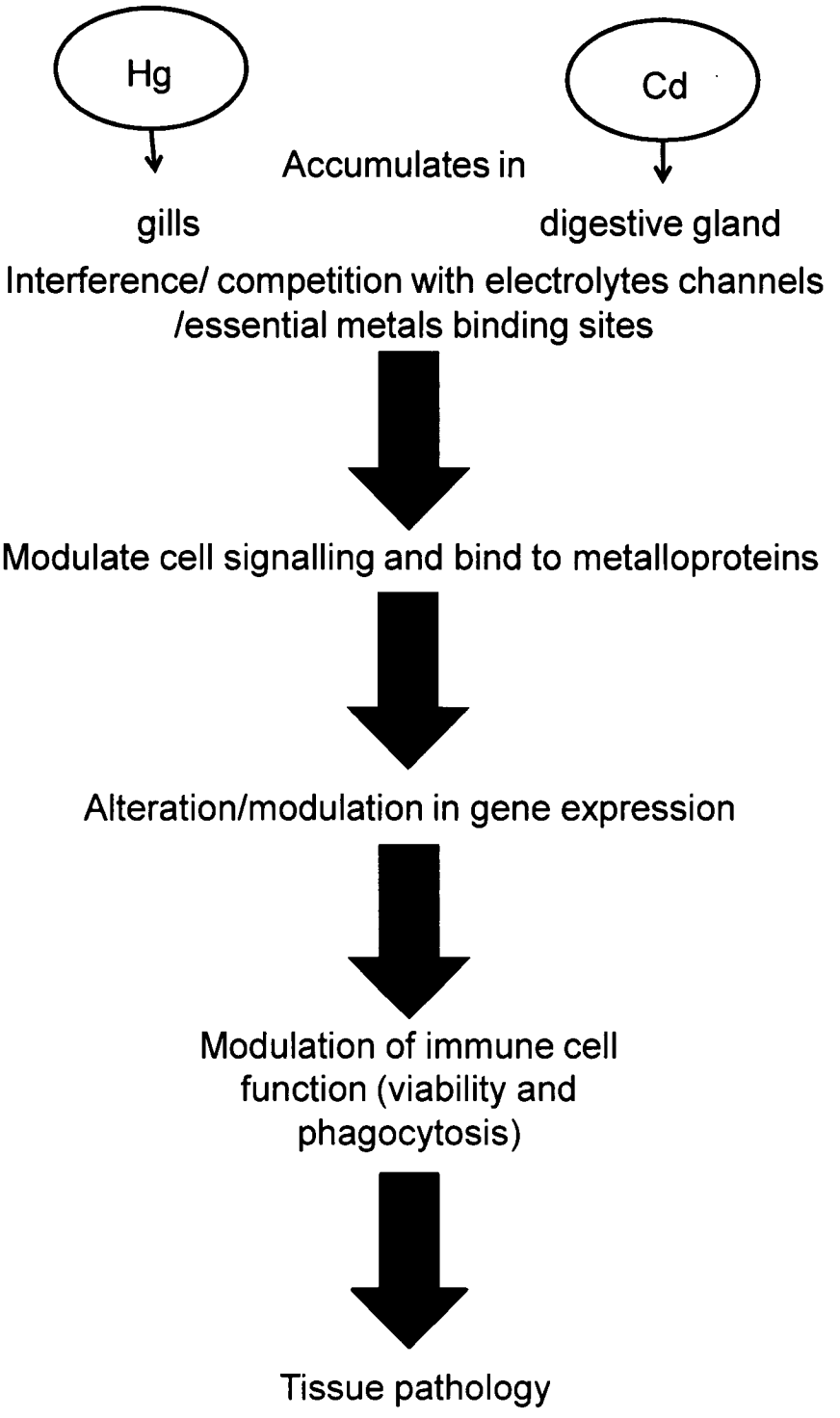

Fig. 8.1 Diagram of Hg and Cd suggested pathways inside the mussels' body cells.

8.8 Future work

One of the most important areas I want to address more deeply was the relationship between the biomarker responses, especially gene expression, and host-parasite interactions. Now that we understand some basic features of the inflammation and immunity of mussels, and also with an LPS challenge; it may be possible to go to the next step of doing experiments with live pathogens and metals. The literature in this area, especially on parasites is weak (e.g., Schuwerack et al., 2007). Work needs to be done to understand these interactions, and how genes are modulated under the effect of stressor (s), in different types of tissues, and at different concentrations of chemical (s). We are a long way from exploring realistic environmental conditions such as multiple exposures, but such basic laboratory experiments are needed.

Another area is the effect of nutritional status on the response to pollution. There are possible correlations between the effect of pollution on feeding status and energy levels, and this could be incorporated into simple scope for growth assays. The availability of energy is especially important for the immune system, given the well known oxygen demand during phagocytosis (respiratory burst). It is likely that both food shortage and pollutant exposure may compound an immunological problem in bivalves, and so a combination of effects should be tested. Ultimately, this will increase our understanding of how pollution leads to disease in aquatic organisms.

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Publications:

1- Sheir, S. K.; Handy, R. D. and Galloway, T. S. (2010): Tissue Injury and Cellular Immune Responses to Mercuric Chloride Exposure in the Common Mussel *Mytilus edulis*: Modulation by Lipopolysaccharide. Ecotoxicology and Environmental Safety. In press.

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